

CORRECTED
VERSION*

BEST AVAILABLE COPY



PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁷ : C12N 15/86, 5/10, A61K 48/00		A1	(11) International Publication Number: WO 00/15820
			(43) International Publication Date: 23 March 2000 (23.03.00)
(21) International Application Number: PCT/US99/20718		(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).	
(22) International Filing Date: 10 September 1999 (10.09.99)		Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>	
(30) Priority Data: 60/099,791 10 September 1998 (10.09.98) US 09/392,822 9 September 1999 (09.09.99) US			
(71) Applicant (for all designated States except US): CALYDON, INC. [US/US]; 1324 Chesapeake Terrace, Sunnyvale, CA 94089 (US).			
(72) Inventors; and (75) Inventors/Applicants (for US only): YU, De, Chao [CN/US]; 1046 Eagle Lane, Foster City, CA 94404 (US). HENDERSON, Daniel, R. [US/US]; 955 Matadero Avenue, Palo Alto, CA 94306 (US).			
(74) Agents: POLIZZI, Catherine, M. et al.; Morrison & Foerster LLP, 755 Page Mill Road, Palo Alto, CA 94304-1018 (US).			
(54) Title: ADENOVIRUS VECTORS CONTAINING CELL STATUS-SPECIFIC RESPONSE ELEMENTS AND METHODS OF USE THEREOF			
<p style="text-align: center;">CN796 HRE PSE E1A E1B</p>			
(57) Abstract			
<p>The present invention provides adenoviral vectors comprising cell status-specific transcriptional regulatory elements which confer cell status-specific transcriptional regulation on an adenoviral gene. A "cell status" is generally a reversible physiological and/or environmental state. The invention further provides compositions and host cells comprising the vectors, as well as methods of using the vectors.</p>			

RECEIVED

JUN 19 2000

Morrison & Foerster, LLP
Palo Alto

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakhstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LI	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

ADENOVIRUS VECTORS CONTAINING CELL STATUS-SPECIFIC RESPONSE ELEMENTS AND METHODS OF USE THEREOF

5 CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims the priority benefit of U.S. Provisional Patent Application No.60/099,791, filed September 10, 1998. The priority application is hereby incorporated herein by reference in its entirety.

10 STATEMENT OF RIGHTS TO INVENTIONS MADE UNDER FEDERALLY SPONSORED RESEARCH (Not Applicable)

TECHNICAL FIELD

15 This invention relates to cell transfection using adenoviral vectors. More specifically, it relates to cell status-specific replication of adenovirus vectors in cells, regardless of tissue or cell type.

BACKGROUND ART

20 In spite of numerous advances in medical research, cancer remains the second leading cause of death in the United States. In the industrialized nations, roughly one in five persons will die of cancer. Traditional modes of clinical care, such as surgical resection, radiotherapy and chemotherapy, have a significant failure rate, especially for solid tumors. Neoplasia resulting in benign tumors can usually be completely cured by removing the mass surgically.
25 If a tumor becomes malignant, as manifested by invasion of surrounding tissue, it becomes much more difficult to eradicate. Once a malignant tumor metastasizes, it is much less likely to be eradicated.

Excluding basal cell carcinoma, there are over one million new cases of cancer per year in the United States alone, and cancer accounts for over one half million deaths per year in this

country. In the world as a whole, the five most common cancers are those of lung, stomach, breast, colon/rectum, and uterine cervix, and the total number of new cases per year is over 6 million.

5 Lung cancer is one of the most refractory of solid tumors because inoperable cases are up to 60% and the 5-year survival is only 13%. In particular, adenocarcinomas, which comprise about one-half of the total lung cancer cases, are mostly chemo-radioresistant. Colorectal cancer is the third most common cancer and the second leading cause of cancer deaths. Pancreatic cancer is virtually always fatal. Thus, current treatment prospects for many patients with these carcinomas are unsatisfactory, and the prognosis is poor.

10 Hepatocellular carcinoma (HCC or malignant hepatoma) is one of the most common cancers in the world, and is especially problematic in Asia. Treatment prospects for patients with hepatocellular carcinoma are dim. Even with improvements in therapy and availability of liver transplant, only a minority of patients are cured by removal of the tumor either by resection or transplantation. For the majority of patients, the current treatments remain
15 unsatisfactory, and the prognosis is poor.

Breast cancer is one of the most common cancers in the United States, with an annual incidence of about 182,000 new cases and nearly 50,000 deaths. In the industrial nations, approximately one in eight women can expect to develop breast cancer. The mortality rate for breast cancer has remained unchanged since 1930. It has increased an average of 0.2% per
20 year, but decreased in women under 65 years of age by an average of 0.3% per year. See e.g., Marchant (1994) *Contemporary Management of Breast Disease II: Breast Cancer*, in: *Obstetrics and Gynecology Clinics of North America* 21:555–560; and Colditz (1993) *Cancer Suppl.* 71:1480–1489.

25 Despite ongoing improvement in the understanding of the disease, breast cancer has remained resistant to medical intervention. Most clinical initiatives are focused on early diagnosis, followed by conventional forms of intervention, particularly surgery and chemotherapy. Such interventions are of limited success, particularly in patients where the tumor has undergone metastasis. There is a pressing need to improve the arsenal of therapies available to provide more precise and more effective treatment in a less invasive way.

Prostate cancer is the fastest growing neoplasm in men with an estimated 244,000 new cases in the United States being diagnosed in 1995, of which approximately 44,000 deaths will result. Prostate cancer is now the most frequently diagnosed cancer in men. Prostate cancer is latent; many men carry prostate cancer cells without overt signs of disease. It is associated with a high morbidity. Cancer metastasis to bone (late stage) is common and is almost always fatal.

Current treatments include radical prostatectomy, radiation therapy, hormonal ablation and chemotherapy. Unfortunately, in approximately 80% of cases, diagnosis of prostate cancer is established when the disease has already metastasized to the bones, thus limiting the effectiveness of surgical treatments. Hormonal therapy frequently fails with time with the development of hormone-resistant tumor cells. Although chemotherapeutic agents have been used in the treatment of prostate cancer, no single agent has demonstrated superiority over its counterparts, and no drug combination seems particularly effective. The generally drug-resistant, slow-growing nature of most prostate cancers makes them particularly unresponsive to standard chemotherapy.

A major, indeed the overwhelming, obstacle to cancer therapy is the problem of selectivity; that is, the ability to inhibit the multiplication of tumor cells, while leaving unaffected the function of normal cells. For example, in prostate cancer therapy, the therapeutic ratio, or ratio of tumor cell killing to normal cell killing of traditional tumor chemotherapy, is only 1.5:1. Thus, more effective treatment methods and pharmaceutical compositions for therapy and prophylaxis of neoplasia are needed.

Solid tumors frequently contain regions that are poorly vascularized, partly because the tumor cells grow faster than the endothelial cells that make up the blood vessels. Tumor cells can remain viable in such hypoxic conditions and are often refractory to chemotherapy and radiation therapy. In a recent study of cervical cancer, the oxygen status of a tumor was shown to be the single most important prognostic factor, ahead of age of patient, menopausal status, clinical stage, size and histology. Hoeckel et al. (1996) *Semin. Radiat. Oncol.* 6:1-8.

Of particular interest is development of more specific, targeted forms of cancer therapy, especially for cancers that are difficult to treat successfully. In contrast to conventional cancer therapies, which result in relatively non-specific and often serious toxicity, more specific

treatment modalities attempt to inhibit or kill malignant cells selectively while leaving healthy cells intact. Radioresistant and chemoresistant tumors present particular challenges, and there is a need for methods of treating these types of tumors.

5 One possible treatment approach for many of these cancers is gene therapy, whereby a gene of interest is introduced into the malignant cell. Various viral vectors, including adenoviral vectors, have been developed as vehicles for gene therapy. The virtually exclusive focus in development of adenoviral vectors for gene therapy is use of adenovirus merely as a vehicle for introducing the gene of interest, not as an effector in itself. Replication of adenovirus has been viewed as an undesirable result, largely due to the host immune response. 10 In the treatment of cancer by replication-defective adenoviruses, the host immune response limits the duration of repeat doses at two levels. First, the capsid proteins of the adenovirus delivery vehicle itself are immunogenic. Second, viral late genes are frequently expressed in transduced cells, eliciting cellular immunity. Thus, the ability to repeatedly administer cytokines, tumor suppressor genes, ribozymes, suicide genes, or genes which convert prodrug 15 to an active drug has been limited by the immunogenicity of both the gene transfer vehicle and the viral gene products of the transfer vehicle as well as the transient nature of gene expression.

Use of adenoviral vectors as therapeutic vehicles for cancer has been reported. Some of these approaches utilize tissue (i.e., cell type) specific transcriptional regulatory elements to 20 selectively drive adenoviral replication (and thus cytotoxicity). U.S. Pat. No. 5,698,443; see also WO 95/11984; WO 96/17053; WO 96/34969; WO 98/35028. While useful and promising, there remain other treatment contexts for which tissue specific replication may be insufficient.

Besides cancerous cells, it is often desirable to selectively destroy certain unwanted 25 cells or tissues. Besides surgery, however, which is invasive, there is a dearth of methods available, particularly non-invasive methods, which would allow such selective cytotoxicity and/or suppression.

There is a need for vector constructs that are capable of eliminating essentially all cancerous cells in a minimum number of administrations before specific immunological 30 response against the vector prevents further treatment and which are suitable for use in

specific, focused cancer ablation treatments. There is also a need for an ability to selectively destroy, or impair, unwanted cells, regardless of cell type and/or regardless of anatomical location.

5

SUMMARY OF THE INVENTION

Replication-competent adenoviral vectors specific for cells in a given, or particular, physiological state that permits or induces expression of polynucleotides under transcriptional control of a cell status-specific TRE, and methods for their use are provided. In these replication-competent adenovirus vectors, one or more adenoviral genes is under transcriptional control of an cell status-specific transcriptional regulatory element (TRE). Preferably, the adenoviral gene under transcriptional control of a cell status-specific TRE is one that is essential for adenoviral propagation. A transgene under control of the cell status-specific TRE may also be present. For the adenoviral vectors of the present invention, a cell status-specific TRE is active in a cell existing in a particular, reversible, physiological state, which may be an aberrant physiological state, i.e., a physiological state that deviates from the typical, or normal, physiological state of that same cell when in a non-dividing or regulated dividing state under normal, physiological conditions.

Accordingly, in one aspect, the invention provides an adenovirus vector comprising an adenovirus gene, wherein said adenovirus gene is under transcriptional control of a cell status-specific TRE. In another embodiment, a cell status-specific TRE is human. In another embodiment, a cell status-specific TRE comprises a cell status-specific promoter and enhancer. In yet another embodiment, a cell status-specific TRE is juxtaposed with a cell type-specific TRE, and together the two elements control expression of an adenovirus gene. Thus, the invention provides adenovirus vectors comprising a TRE comprising a cell status-specific TRE and a cell type-specific TRE.

In some embodiments, the adenovirus gene under transcriptional control of a cell status-specific TRE is an adenovirus gene essential for replication. In some embodiments, the

adenoviral gene essential for replication is an early gene. In another embodiment, the early gene is E1A. In another embodiment, the early gene is E1B. In yet another embodiment, both E1A and E1B are under transcriptional control of a cell status-specific TRE. In other embodiments, the adenovirus gene essential for replication is a late gene.

5 In another embodiment, the cell status-specific TRE comprises a hypoxia responsive element. In another embodiment, the cell status-specific TRE comprises the nucleotide sequence of SEQ ID NO:1.

 In another embodiment, the cell status-specific TRE comprises a cell cycle-specific TRE. The cell cycle-specific TRE can be derived from the E2F1 5' flanking region. In one
10 embodiment, the cell cycle-specific TRE comprises the nucleotide sequence depicted in SEQ ID NO:2.

 In other embodiments, the adenovirus vector can further comprise a transgene, wherein said transgene is under transcriptional control of an cell status-specific TRE. In some embodiments, the transgene is a cytotoxic gene.

15 In other embodiments, the adenoviral vector comprises an adenoviral gene essential for adenoviral replication under control of a first cell status-specific TRE, and a second adenoviral gene essential for adenoviral replication under control of a second cell status-specific TRE. The first and the second cell status-specific TREs can be identical, substantially identical, or different from, one another.

20 In other embodiments, the adenoviral vector comprises an adenoviral gene essential for adenoviral replication under control of a first cell status-specific TRE, and a transgene under control of a second cell status-specific TRE. The first and the second cell status-specific TREs can be substantially identical to, or different from, one another.

 In other embodiments, the adenovirus vector comprises an adenovirus gene under
25 transcriptional control of a cell status-specific TRE, and a second adenovirus gene under transcriptional control of a cell type-specific TRE. In other embodiments, the adenovirus vector comprises an adenovirus gene under transcriptional control of a cell status-specific TRE, and a transgene under transcriptional control of a cell type-specific TRE.

30 In another aspect, the invention provides a host cell comprising the adenovirus vector(s) described herein.

In another aspect, the invention provides pharmaceutical compositions comprising an adenovirus vector(s) described herein.

In another aspect, the invention provides kits which contain an adenoviral vector(s) described herein.

5 In another aspect, methods are provided for conferring selective cytotoxicity in target cells (i.e., cells which permit or induce a cell status-specific TRE to function), comprising contacting the cells with an adenovirus vector(s) described herein, whereby the vector enters the cell.

10 Another embodiment of the invention is an adenovirus which replicates preferentially in mammalian cells whose cell status permits or induces the function of a cell status-specific TRE.

 In another aspect, methods are provided for propagating an adenovirus specific for mammalian cells whose cell status permits the function of a cell status-specific TRE, said method comprising combining an adenovirus vector(s) described herein with mammalian cells
15 whose cell status permits the function of a cell status-specific TRE, whereby said adenovirus is propagated.

 The invention further provides methods of suppressing tumor cell growth, more particularly a target tumor cell (i.e., a tumor cell that permits or induces a cell status-specific TRE to function), comprising contacting a tumor cell with an adenoviral vector of the
20 invention such that the adenoviral vector enters the tumor cell and exhibits selective cytotoxicity for the tumor cell.

 In another aspect, methods are provided for detecting cells whose cell status permits the function of a cell status-specific TRE in a biological sample, comprising contacting cells of a biological sample with an adenovirus vector(s) described herein, and detecting replication of
25 the adenovirus vector, if any.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic representation of adenovirus vector CN796, in which the E1A gene is under transcriptional control of an HRE and a PSA-TRE, as described in Example 1.

Figure 2 shows the nucleotide sequence of an HRE from the 5' flanking region of a rat enolase-1 gene (SEQ ID NO:1).

Figure 3 shows the nucleotide sequence of the 5' flanking region of a human E2F1 gene (SEQ ID NO:2). The asterisk indicates the transcription start site.

5 Figure 4 depicts a nucleotide sequence of a prostate-specific antigen TRE.

Figure 5 depicts a nucleotide sequence of a carcinoembryonic antigen TRE.

Figure 6 depicts a nucleotide sequence of a human glandular kallikrein TRE.

Figure 7 depicts a nucleotide sequence of a mucin TRE.

Figure 8 depicts a nucleotide sequence of a rat probasin TRE.

10 Figure 9 depicts a nucleotide sequence and translated amino acid sequence of an adenovirus death protein.

MODES FOR CARRYING OUT THE INVENTION

15 We have discovered and constructed replication-competent adenovirus vectors which contain an adenoviral gene under transcriptional control of a cell status-specific transcriptional response element (TRE) such that the adenovirus gene is transcribed preferentially in cells whose cell status permit the function of the cell status-specific TRE, and have developed methods using these adenovirus vectors. In some preferred embodiments, the adenovirus vectors of this invention comprise at least one adenovirus gene necessary for adenoviral replication, preferably at least one early gene, under the transcriptional control of a TRE specifically regulated by binding of transcriptional factor(s) and/or co-factor(s) necessary for transcription regulated by the cell status-specific TRE. By providing for cell status-specific transcription of at least one adenovirus gene required for replication, the invention provides adenovirus vectors that can be used for specific cytotoxic effects due to selective replication and/or selective transcription. This is especially useful in the cancer context, in which targeted cell killing is desirable. This is also useful for targeted cytotoxic effects in other, non-tumor cells, when selective destruction and/or suppression of these cells is desirable. The vectors can also be useful for detecting the presence of cells whose cell status permits function of a cell status-specific TRE in, for example, an appropriate biological (such as clinical) sample.

20

25

Further, the adenovirus vector(s) can optionally selectively produce one or more proteins of interest in a target cell by using a cell status-specific TRE.

We have found that adenovirus vectors of the invention replicate and/or express an adenoviral gene operably linked to a cell status-specific TRE preferentially in cells whose status permits the function of a cell status-specific TRE. In contrast to previously-described adenoviral vectors designed to replicate preferentially in specific, differentiated cell types, the adenovirus vectors of the present invention comprise regulatory elements that are not cell type-specific. Rather, they confer cell status-specific adenoviral replication and/or cell status-specific expression of an operably linked adenoviral gene and/or transgene.

The adenovirus vectors of the present invention comprise a cell status-specific TRE which is functional in a cell which exhibits a particular physiological (i.e., environmental or metabolic) characteristic which is reversible and/or progressive. The target cell may exhibit an aberrant physiological state, such as low oxygen tension, or may be subjected to an aberrant environmental condition, such as heat or ionizing radiation, in order for the cell-status TRE to function. The replication preference of these vectors is indicated by comparing the level of replication (i.e., titer) in cells in a requisite physiological state or condition (for example, an aberrant physiological state) to the level of replication in cells not exhibiting the requisite physiological state (for example, under normal physiological conditions). Thus, the invention also uses and takes advantage of what has been considered an undesirable aspect of adenoviral vectors, namely, their replication and possibly concomitant immunogenicity. The probability of runaway infection is significantly reduced due to the cell status-specific requirements for viral replication. Without wishing to be bound by any particular theory, the inventors note that production of adenovirus proteins can serve to activate and/or stimulate the immune system, generally and/or specifically toward target cells producing adenoviral proteins, which can be an important consideration in the cancer context, where patients are often moderately to severely immunocompromised.

The adenovirus vectors of the present invention find particular utility in specific treatment regimens, in which the treatment is highly focused toward, for example, a particular cancer which might otherwise be inoperable or untreatable. An important feature of the

invention is that the vectors are useful in these treatments regardless of the tissue or cell type of the cancer, and yet their cytotoxicity can be targeted to certain locations.

General Techniques

5 The practice of the present invention will employ, unless otherwise indicated, conventional techniques of molecular biology (including recombinant techniques), microbiology, cell biology, biochemistry, and immunology, which are within the skill of the art. Such techniques are explained fully in the literature, such as, "Molecular Cloning: A Laboratory Manual", second edition (Sanbrook et al., 1989); "Oligonucleotide Synthesis" (M.J. Gait, ed., 1984); "Animal Cell Culture" (R.I. Freshney, ed., 1987); "Methods in Enzymology" (Academic Press, Inc.); "Handbook of Experimental Immunology" (D.M. Wei & C.C. Blackwell, eds.); "Gene Transfer Vectors for Mammalian Cells" (J.M. Miller & M.P. Calos, eds., 1987); "Current Protocols in Molecular Biology" (F.M. Ausubel et al., eds., 1987); "PCR: The Polymerase Chain Reaction", (Mullis et al., eds., 1994); "Current Protocols in Immunology" (J.E. Coligan et al., eds., 1991).

15 For techniques related to adenovirus, see, *inter alia*, Felgner and Ringold (1989) *Nature* 337:387-388; Berkner and Sharp (1983) *Nucl. Acids Res.* 11:6003-6020; Graham (1984) *EMBO J.* 3:2917-2922; Bett et al. (1993) *J. Virology* 67:5911-5921; Bett et al. (1994) *Proc. Natl. Acad. Sci. USA* 91:8802-8806.

Definitions

20 As used herein, a "transcription response element" or "transcriptional regulatory element", or "TRE" is a polynucleotide sequence, preferably a DNA sequence, which increases transcription of an operably linked polynucleotide sequence in a host cell that allows that TRE to function. A TRE can comprise an enhancer and/or a promoter.

25 As used herein, the term "cell status-specific TRE" is one that confers transcriptional activation on an operably linked polynucleotide in a cell which allows a cell status-specific TRE to function, i.e., a cell which exhibits a particular physiological condition, including, but not limited to, an aberrant physiological state. "Cell status" thus refers to a given, or particular, physiological state (or condition) of a cell, which is reversible and/or progressive. The physiological state may be generated internally or externally; for example, it may be a

metabolic state (such as low oxygen), or it may be generated due to heat or ionizing radiation. "Cell status" is distinct from a "cell type", which relates to a differentiation state of a cell, which under normal conditions is irreversible. Generally (but not necessarily), as discussed herein, a cell status is embodied in an aberrant physiological state, examples of which are given below.

A "normal cell status" or "normal physiological state" is the status of a cell which exists in normal physiological conditions and which is non-dividing or divides in a regulated manner, i.e., a cell in a normal physiological state.

The terms "aberrant cell status" and "aberrant physiological state", used interchangeably herein, intend a condition of a cell which is a response to, a result of, or is influenced by, an aberrant physiological condition. An aberrant cell status is neither cell type-specific nor tissue type-specific. An aberrant cell status is defined in relation to a cell of the same type which is in a non-dividing/regulated dividing state and under normal physiological conditions.

"Normal physiological conditions" are known to those skilled in the art. These conditions may vary, depending on a cell's location in the body. For example, oxygen tension can vary from tissue to tissue. For *in vitro* analyses for the purposes of determining whether a TRE is responsive to deviations from normal physiological conditions, these conditions generally include an oxygen concentration of about 20% O₂, and a temperature of about 37°C.

"Regulated cell division" is a term well understood in the art and refers to the normal mitotic activity of a cell. Those skilled in the art understand that normal mitotic activity varies from cell type to cell type. For example, many terminally differentiated cells in tissues exhibit little or no mitotic activity, while hematopoietic cells are generally mitotically active.

An "aberrant physiological condition" or "aberrant physiological state", as used herein, intends a condition which deviates from normal physiological conditions, and includes, but is not limited to, a physiological condition that is characterized by alterations in oxygen concentration, such as hypoxic conditions; temperatures which deviate from physiological temperatures; a condition that triggers apoptosis; radiation, including ionizing radiation and UV irradiation; de-regulated cell division, resulting for example, from a lack of, or insufficient amounts of, or inactivity of, a factor which controls cell division, such as, for example,

retinoblastoma protein (Rb); variations in timing of cell cycle; infection with a pathogen; exposure to a chemical substance; or a combination of the above-listed conditions. Another example is a mutation that could, or does, exist in any cell type, i.e., its existence does not depend on, or is not related to, the differentiation state of the cell.

5 A "target cell", as used herein, is one that permits or induces the function of a cell status-specific TRE such that it effects transcriptional activation of an operably linked polynucleotide. A target cell is one which exhibits a requisite physiological (or environmental) state, which may be an aberrant physiological state. Preferably, a target cell is a mammalian cell, preferably a human cell. A target cell may or may not be neoplastic. By
10 transcriptional activation, it is intended that transcription is increased in the target cell above the levels in a control cell (e.g., a that cell when not exhibiting a requisite physiological state (generally a normal physiological state) by at least about 2 fold, preferably at least about 5 fold, preferably at least about 10 fold, more preferably at least about 20 fold, more preferably at least about 50 fold, more preferably at least about 100 fold, more preferably at least about
15 200 fold, even more preferably at least about 400 fold to about 500 fold, even more preferably at least about 1000 fold. The normal levels are generally the level of activity (if any) in a cell as tested under conditions that activate the cell status-specific TRE, or the level of activity (if any) of a reporter construct lacking a cell status-specific TRE as measured in a cell exhibiting the requisite physiological condition.

20 A "functionally-preserved" variant of a cell status-specific TRE is a cell status-specific TRE which differs from another cell status-specific TRE, but still retains cell status cell-specific transcription activity. The difference in an cell status-specific TRE can be due to differences in linear sequence, arising from, for example, single base mutation(s), addition(s), deletion(s), and/or modification(s) of the bases. The difference can also arise from changes in
25 the sugar(s), and/or linkage(s) between the bases of a cell status-specific TRE.

 An "adenovirus vector" or "adenoviral vector" (used interchangeably) comprises a polynucleotide construct of the invention. A polynucleotide construct of this invention may be in any of several forms, including, but not limited to, DNA, DNA encapsulated in an adenovirus coat, DNA packaged in another viral or viral-like form (such as herpes simplex, and AAV), DNA encapsulated in liposomes, DNA complexed with polylysine, complexed
30

with synthetic polycationic molecules, conjugated with transferrin, and complexed with compounds such as PEG to immunologically "mask" the molecule and/or increase half-life, and conjugated to a nonviral protein. Preferably, the polynucleotide is DNA. As used herein, "DNA" includes not only bases A, T, C, and G, but also includes any of their analogs or modified forms of these bases, such as methylated nucleotides, internucleotide modifications such as uncharged linkages and thioates, use of sugar analogs, and modified and/or alternative backbone structures, such as polyamides. For purposes of this invention, adenovirus vectors are replication-competent in a target cell.

The terms "polynucleotide" and "nucleic acid", used interchangeably herein, refer to a polymeric form of nucleotides of any length, either ribonucleotides or deoxyribonucleotides. These terms include a single-, double- or triple-stranded DNA, genomic DNA, cDNA, RNA, DNA-RNA hybrid, or a polymer comprising purine and pyrimidine bases, or other natural, chemically, biochemically modified, non-natural or derivatized nucleotide bases. The backbone of the polynucleotide can comprise sugars and phosphate groups (as may typically be found in RNA or DNA), or modified or substituted sugar or phosphate groups. Alternatively, the backbone of the polynucleotide can comprise a polymer of synthetic subunits such as phosphoramidates and thus can be a oligodeoxynucleoside phosphoramidate (P-NH₂) or a mixed phosphoramidate- phosphodiester oligomer. Peyrottes et al. (1996) *Nucleic Acids Res.* 24: 1841-8; Chaturvedi et al. (1996) *Nucleic Acids Res.* 24: 2318-23; Schultz et al. (1996) *Nucleic Acids Res.* 24: 2966-73. A phosphorothiate linkage can be used in place of a phosphodiester linkage. Braun et al. (1988) *J. Immunol.* 141: 2084-9; Latimer et al. (1995) *Mol. Immunol.* 32: 1057-1064. In addition, a double-stranded polynucleotide can be obtained from the single stranded polynucleotide product of chemical synthesis either by synthesizing the complementary strand and annealing the strands under appropriate conditions, or by synthesizing the complementary strand de novo using a DNA polymerase with an appropriate primer.

The following are non-limiting examples of polynucleotides: a gene or gene fragment, exons, introns, mRNA, tRNA, rRNA, ribozymes, cDNA, recombinant polynucleotides, branched polynucleotides, plasmids, vectors, isolated DNA of any sequence, isolated RNA of any sequence, nucleic acid probes, and primers. A polynucleotide may comprise modified

nucleotides, such as methylated nucleotides and nucleotide analogs, uracyl, other sugars and linking groups such as fluororibose and thioate, and nucleotide branches. The sequence of nucleotides may be interrupted by non-nucleotide components. A polynucleotide may be further modified after polymerization, such as by conjugation with a labeling component.

5 Other types of modifications included in this definition are caps, substitution of one or more of the naturally occurring nucleotides with an analog, and introduction of means for attaching the polynucleotide to proteins, metal ions, labeling components, other polynucleotides, or a solid support. Preferably, the polynucleotide is DNA. As used herein, "DNA" includes not only bases A, T, C, and G, but also includes any of their analogs or modified forms of these bases,
10 such as methylated nucleotides, internucleotide modifications such as uncharged linkages and thioates, use of sugar analogs, and modified and/or alternative backbone structures, such as polyamides.

A polynucleotide or polynucleotide region has a certain percentage (for example, 80%, 85%, 90%, or 95%) of "sequence identity" to another sequence means that, when aligned, that
15 percentage of bases are the same in comparing the two sequences. This alignment and the percent homology or sequence identity can be determined using software programs known in the art, for example those described in *Current Protocols in Molecular Biology* (F.M. Ausubel et al., eds., 1987) Supplement 30, section 7.7.18, Table 7.7.1. A preferred alignment program is ALIGN Plus (Scientific and Educational Software, Pennsylvania), preferably using default
20 parameters.

"Under transcriptional control" is a term well-understood in the art and indicates that transcription of a polynucleotide sequence, usually a DNA sequence, depends on its being operably (operatively) linked to an element which contributes to the initiation of, or promotes, transcription. "Operably linked" refers to a juxtaposition wherein the elements are in an
25 arrangement allowing them to function.

"Replication" and "propagation" are used interchangeably and refer to the ability of a polynucleotide construct of the invention to reproduce, or proliferate. This term is well understood in the art. For purposes of this invention, replication involves production of adenovirus proteins and is generally directed to reproduction of adenovirus. Replication can

be measured using assays standard in the art and described herein, such as a burst assay, plaque assay, or a one-step growth curve assay.

As used herein, "cytotoxicity" is a term well understood in the art and refers to a state in which a cell's usual biochemical or biological activities are compromised (i.e., inhibited).

5 These activities include, but are not limited to, metabolism; cellular replication; DNA replication; transcription; translation; uptake of molecules. "Cytotoxicity" includes cell death and/or cytolysis. Assays are known in the art which indicate cytotoxicity, such as dye exclusion, ³H-thymidine uptake, and plaque assays.

10 The term "selective cytotoxicity", as used herein, refers to the cytotoxicity conferred by an adenovirus vector of the present invention on a cell which allows or induces a cell status-specific TRE to function (a target cell) when compared to the cytotoxicity conferred by an adenoviral vector of the present invention on a cell which does not allow a cell status-specific TRE to function (a non-target cell). Such cytotoxicity may be measured, for example, by plaque assays, by reduction or stabilization in size of a tumor comprising target cells, or the
15 reduction or stabilization of serum levels of a marker characteristic of the tumor cells, or a tissue-specific marker, e.g., a cancer marker, such as prostate specific antigen.

In the context of adenovirus, a "heterologous polynucleotide" or "heterologous gene" or "transgene" is any polynucleotide or gene that is not present in wild-type adenovirus. Preferably, the transgene will also not be expressed or present in the target cell prior to
20 introduction by the adenovirus vector. Examples of preferred transgenes are provided below.

In the context of adenovirus, a "heterologous" promoter or enhancer is one which is not associated with or derived from an adenovirus gene.

In the context of adenovirus, an "endogenous" promoter, enhancer, or TRE is native to or derived from adenovirus.

25 In the context of a cell status-specific TRE, a "heterologous" promoter or enhancer is one which is not normally associated in a cell with or derived from a cell status-specific TRE. Examples of a heterologous promoter or enhancer are the albumin promoter or enhancer and other viral promoters and enhancers, such as SV40, or cell type-specific TREs such as a prostate-specific TRE.

A "cell type-specific TRE" is preferentially functional in a specific type of cell relative to other types of cells. In contrast to cell status, "cell type" is a reflection of a differentiation state of a cell which is irreversible. For example, a prostate-specific antigen is expressed in prostate cells, but is not substantially expressed in other cell types such as hepatocytes, astrocytes, cardiocytes, lymphocytes, etc. Generally, a cell type-specific TRE is active in only one cell type. When a cell type-specific TRE is active in more than one cell type, its activity is restricted to a limited number of cell types, i.e., it is not active in all cell types. A cell type-specific TRE may or may not be tumor cell specific.

"Suppressing" tumor growth indicates a growth state that is curtailed when compared to growth without contact with, i.e., transfection by, an adenoviral vector described herein. Tumor cell growth can be assessed by any means known in the art, including, but not limited to, measuring tumor size, determining whether tumor cells are proliferating using a ^3H -thymidine incorporation assay, or counting tumor cells. "Suppressing" tumor cell growth means any or all of the following states: slowing, delaying, and stopping tumor growth, as well as tumor shrinkage.

As used herein, the terms "neoplastic cells", "neoplasia", "tumor", "tumor cells", "cancer" and "cancer cells", (used interchangeably) refer to cells which exhibit relatively autonomous growth, so that they exhibit an aberrant growth phenotype characterized by a significant loss of control of cell proliferation (i.e., de-regulated cell division). Neoplastic cells can be malignant or benign.

A "host cell" includes an individual cell or cell culture which can be or has been a recipient of an adenoviral vector(s) of this invention. Host cells include progeny of a single host cell, and the progeny may not necessarily be completely identical (in morphology or in total DNA complement) to the original parent cell due to natural, accidental, or deliberate mutation and/or change. A host cell includes cells transfected or infected *in vivo* or *in vitro* with an adenoviral vector of this invention.

"Replication" and "propagation" are used interchangeably and refer to the ability of an adenovirus vector of the invention to reproduce or proliferate. These terms are well understood in the art. For purposes of this invention, replication involves production of adenovirus proteins and is generally directed to reproduction of adenovirus. Replication can

be measured using assays standard in the art and described herein, such as a burst assay or plaque assay. "Replication" and "propagation" include any activity directly or indirectly involved in the process of virus manufacture, including, but not limited to, viral gene expression; production of viral proteins, nucleic acids or other components; packaging of viral components into complete viruses; and cell lysis.

An "ADP coding sequence" is a polynucleotide that encodes ADP or a functional fragment thereof. In the context of ADP, a "functional fragment" of ADP is one that exhibits cytotoxic activity, especially cell lysis, with respect to adenoviral replication. Ways to measure cytotoxic activity are known in the art and are described herein.

A polynucleotide that "encodes" an ADP polypeptide is one that can be transcribed and/or translated to produce an ADP polypeptide or a fragment thereof. The anti-sense strand of such a polynucleotide is also said to encode the sequence.

An "ADP polypeptide" is a polypeptide containing at least a portion, or region, of the amino acid sequence of an ADP (see, for example, SEQ ID NO:5), and which displays a function associated with ADP, particularly cytotoxicity, more particularly, cell lysis. As discussed herein, these functions can be measured using techniques known in the art. It is understood that certain sequence variations may be used, due to, for example, conservative amino acid substitutions, which may provide ADP polypeptides.

A polynucleotide sequence that is "depicted in" a SEQ ID NO means that the sequence is present as an identical contiguous sequence in the SEQ ID NO. The term encompasses portions, or regions of the SEQ ID NO as well as the entire sequence contained within the SEQ ID NO.

A "biological sample" encompasses a variety of sample types obtained from an individual and can be used in a diagnostic or monitoring assay. The definition encompasses blood and other liquid samples of biological origin, solid tissue samples such as a biopsy specimen or tissue cultures or cells derived therefrom, and the progeny thereof. The definition also includes samples that have been manipulated in any way after their procurement, such as by treatment with reagents, solubilization, or enrichment for certain components, such as proteins or polynucleotides. The term "biological sample" encompasses a clinical sample, and

also includes cells in culture, cell supernatants, cell lysates, serum, plasma, biological fluid, and tissue samples.

An "individual" is a vertebrate, preferably a mammal, more preferably a human. Mammals include, but are not limited to, farm animals, sport animals, rodents, primates, and
5 pets.

An "effective amount" is an amount sufficient to effect beneficial or desired results, which may include clinical results. An effective amount can be administered in one or more administrations. For purposes of this invention, an effective amount of an adenoviral vector is an amount that is sufficient to palliate, ameliorate, stabilize, reverse, slow or delay the
10 progression of the disease state.

Adenoviral vectors comprising a cell status-specific TRE

The present invention provides adenoviral vector constructs which comprise an adenovirus gene under transcriptional control of a cell status-specific TRE. Preferably, the adenovirus gene contributes to cytotoxicity (whether direct and/or indirect), more preferably
15 one that contributes to or causes cell death, even more preferably is essential for adenoviral replication. Examples of a gene that contributes to cytotoxicity include, but are not limited to, adenovirus death protein (ADP; discussed below). When the adenovirus vector(s) is selectively (i.e., preferentially) replication competent for propagation in target cells, i.e., cells which permit or induce a cell-status TRE to function, these cells will be preferentially killed
20 upon adenoviral proliferation. Once the target cells are destroyed due to selective cytotoxic and/or cytolytic replication, the adenovirus vector replication is significantly reduced, thus lessening the probability of runaway infection and undesirable bystander effects. *In vitro* cultures may be retained to monitor the mixture (such as, for example, a biopsy or other appropriate biological sample) for occurrence (i.e., presence) and/or recurrence of the target
25 cell, e.g., a neoplastic cell or other undesired cell. To further ensure cytotoxicity, one or more transgenes having a cytotoxic effect may also be present and under selective transcriptional control. In this embodiment, one may provide higher confidence that the target cells will be destroyed. Additionally, or alternatively, an adenovirus gene that contributes to cytotoxicity

and/or cell death (such as ADP) may be included in the adenoviral vector, either free of, or under, selective transcriptional control.

Cell status-specific TREs for use in the adenoviral vectors of the present invention can be derived from any species, preferably a mammal. A number of genes have been described which are expressed in response to, or in association with, a cell status. Any of these cell status-associated genes may be used to generate a cell status-specific TRE.

An example of a cell status is cell cycle. An exemplary gene whose expression is associated with cell cycle is E2F-1, a ubiquitously expressed, growth-regulated gene, which exhibits peak transcriptional activity in S phase. Johnson et al. (1994) *Genes Dev.* 8:1514-1525. The RB protein, as well as other members of the RB family, form specific complexes with E2F-1, thereby inhibiting its ability to activate transcription. Thus, E2F-1-responsive promoters are down-regulated by RB. Many tumor cells have disrupted RB function, which can lead to de-repression of E2F-1-responsive promoters, and, in turn, de-regulated cell division.

Accordingly, in one embodiment, the invention provides an adenoviral vector in which an adenoviral gene (preferably a gene necessary for replication) is under transcriptional control of a cell status-specific TRE, wherein the cell status-specific TRE comprises a cell cycle-activated, or cell-cycle specific, TRE. In one embodiment, the cell cycle-activated TRE is an E2F1 TRE. In one embodiment, this TRE comprises the sequence depicted in Figure 3 and SEQ ID NO:2.

Another group of genes which are regulated by cell status are those whose expression is increased in response to hypoxic conditions. Bunn and Poyton (1996) *Physiol. Rev.* 76:839-885; Dachs and Stratford (1996) *Br. J. Cancer* 74:5126-5132; Guillemin and Krasnow (1997) *Cell* 89:9-12. Many tumors have insufficient blood supply, due in part to the fact that tumor cells typically grow faster than the endothelial cells that make up the blood vessels, resulting in areas of hypoxia in the tumor. Folkman (1989) *J. Natl. Cancer Inst.* 82:4-6; and Kallinowski (1996) *The Cancer J.* 9:37-40. An important mediator of hypoxic responses is the transcriptional complex HIF-1, or hypoxia inducible factor-1, which interacts with a hypoxia-responsive element (HRE) in the regulatory regions of several genes, including vascular endothelial growth factor, and several genes encoding glycolytic enzymes, including enolase-

1. Murine HRE sequences have been identified and characterized. Firth et al. (1994) *Proc. Natl. Acad. Sci. USA* 91:6496-6500. An HRE from a rat enolase-1 promoter is described in Jiang et al. (1997) *Cancer Res.* 57:5328-5335. An HRE from a rat enolase-1 promoter is depicted in Figure 2 and given as SEQ ID NO:1.

5 Accordingly, in one embodiment, an adenovirus vector comprises an adenovirus gene, preferably an adenoviral gene essential for replication, under transcriptional control of a cell status-specific TRE comprising an HRE. In one embodiment, the cell status-specific TRE comprises the HRE depicted in Figure 2 and SEQ ID NO:1.

10 Other cell status-specific TREs include heat-inducible (i.e., heat shock) promoters, and promoters responsive to radiation exposure, including ionizing radiation and UV radiation. For example, the promoter region of the early growth response-1 (Egr-1) gene contains an element(s) inducible by ionizing radiation. Hallahan et al. (1995) *Nat. Med.* 1:786-791; and Tsai-Morris et al. (1988) *Nucl. Acids. Res.* 16:8835-8846. Heat-inducible promoters, including heat-inducible elements, have been described. See, for example Welsh (1990) in "Stress
15 Proteins in Biology and Medicine", Morimoto, Tisseries, and Georgopoulos, eds. Cold Spring Harbor Laboratory Press; and Perisic et al. (1989) *Cell* 59:797-806. Accordingly, in some embodiments, the cell status-specific TRE comprises an element(s) responsive to ionizing radiation. In one embodiment, this TRE comprises a 5' flanking sequence of an Egr-1 gene. In other embodiments, the cell status-specific TRE comprises a heat shock responsive, or heat-
20 inducible, element.

 A cell status-specific TRE can also comprise multimers. For example, an HRE can comprise a tandem series of at least two, at least three, at least four, or at least five hypoxia-responsive elements. These multimers may also contain heterologous promoter and/or enhancer sequences.

25 A cell status-specific TRE may or may not lack a silencer. The presence of a silencer (i.e., a negative regulatory element) may assist in shutting off transcription (and thus replication) in non-permissive cells (i.e., cell in a normal cell state). Thus, presence of a silencer may confer enhanced cell status-specific replication by more effectively preventing adenoviral vector replication in non-target cells. Alternatively, lack of a silencer may assist in

effecting replication in target cells, thus conferring enhanced cell status-specific replication due to more effective replication in target cells.

In other embodiments, the adenoviral vector comprises an adenoviral gene essential for adenoviral replication under control of a first cell status-specific TRE, and a second adenoviral gene essential for adenoviral replication under control of a second cell status-specific TRE.

The first and the second cell status-specific TREs may or may not be identical, and may or may not be substantially identical to one another. By "substantially identical" is meant a requisite degree of sequence identity between the two TREs. The degree of sequence identity between these TREs is at least about 80%, preferably at least about 85%, more preferably at least about 90%, even more preferably at least about 95%, even more preferably at least about 98%, and most preferably 100%. Sequence identity can be determined by a sequence comparison using, i.e., sequence alignment programs that are known in the art, such as those described in *Current Protocols in Molecular Biology* (F.M. Ausubel et al., eds., 1987) Supplement 30, section 7.7.18, Table 7.7.1 A preferred alignment program is ALIGN Plus (Scientific and Educational Software, Pennsylvania), preferably using default parameters. Alternatively, hybridization under stringent conditions can also indicate degree of sequence identity. Stringent conditions are known in the art; an example of a stringent condition is 80°C (or higher temperature) and 6 X SSC (or less concentrated SSC). Other hybridization conditions and parameters (in order of increasing stringency) are: incubation temperatures of 25°C, 37°C, 50°C, and 68°C; buffer concentrations of 10 X SSC, 6 X SSC, 1 X SSC, 0.1 X SSC (where 1 X SSC is 0.15 M NaCl and 15 mM citrate buffer) and their equivalents using other buffer systems; formamide concentrations of 0%, 25%, 50%, and 75%; incubation times from about 24 hours about 5 minutes; 1, 2, or more washing steps; wash incubation times of 1, 2, or 15 minutes; and wash solutions of 6 X SSC, 1 X SSC, 0.1 X SSC, or deionized water.

Adenoviral constructs in which the first and second cell status-specific TREs are identical or substantially identical, particularly if these TREs control transcription of early genes (such as E1A and E1B), may display an instability which may be desirable in certain contexts, such as when an automatic "self-destruction" property can shut down the virus, thereby controlling the degree of propagation. Accordingly, in some embodiments, the first and second cell status-specific TRE, or the first and second TRE (one of which is a cell-status-

specific TRE) are sufficiently identical to confer instability when compared to two TREs which are less identical with respect to each other (i.e., have more sequence divergence or dissimilarity). Preferred embodiments are those in which the two TREs control E1A and E1B respectively. "Instability" means that the structural integrity of the adenoviral vectors is not preserved as the virus replicates in cells, and can be measured using standard methods in the art, such as Southern analysis. In other embodiments, the first and second TREs are sufficiently divergent and/or placed in the vector such that the vector is stable (i.e., the structural integrity of the adenoviral vector is preserved).

In other embodiments, the adenoviral vector comprises an adenoviral gene essential for adenoviral replication under control of a first cell status-specific TRE, and a transgene under control of a second cell status-specific TRE. The first and the second cell status-specific TREs may or may not be substantially identical to one another.

In some embodiments, a cell status-specific TRE can be juxtaposed with another TRE, such as a different cell status-specific TRE, or, alternatively, a cell type-specific TRE.

"Juxtaposed" means a cell status-specific TRE and the second TRE transcriptionally control the same gene, or at least are proximate with respect to the same gene. For these embodiments, the cell status-specific TRE and the second TRE may be in any of a number of configurations, including, but not limited to, (a) next to each other (i.e., abutting); (b) both 5' to the gene that is transcriptionally controlled (i.e., may have intervening sequences between them); (c) one TRE 5' and the other TRE 3' to the gene. For example, as described in Example 1 and shown in Figure 1, a cell type-specific TRE can be juxtaposed with a cell status-specific TRE to control transcription of an operably linked adenoviral gene. Such "composite" TREs can be used to confer both cell status- and cell type-specific expression of an operably linked polynucleotide, and thus may confer significantly greater specificity and/or efficacy. Examples of cell type-specific TREs are provided below. Alternatively, "composite" TREs can be used to confer different, and possibly synergistic, cell status specificity. For example, a composite TRE could confer specificity to hypoxia and heat shock.

Example 1 provides a description of an adenovirus construct in which a composite TRE upstream of E1A consisting of an HRE and a prostate-specific TRE, PSA-TRE (which consists of enhancer sequences -5322 to -3738 fused to PSA promoter sequence -541 to +12; see U.S.

Pat. Nos. 5,871,726; 5,648,478). Accordingly, in some embodiments, the invention provides an adenovirus vector comprising an adenovirus gene essential for replication, preferably an early gene, preferably E1A or E1B, under transcriptional control of a TRE comprising an HRE (preferably comprising or consisting of the 67-base fragment depicted in SEQ ID NO:1) and a prostate cell specific TRE, preferably comprising a PSA enhancer (preferably -5322 to -3738; or about 503 to about 2086 of SEQ ID NO:3 (bases about 503 to about 2086 of Figure 4), and a promoter, preferably comprising a PSA enhancer and a PSA promoter (about 5285 to about 5836 of SEQ ID NO:3).

As is readily appreciated by one skilled in the art, a cell status-specific TRE is a polynucleotide sequence, and, as such, can exhibit function over a variety of sequence permutations. Methods of nucleotide substitution, addition, and deletion are known in the art, and readily available functional assays (such as the CAT or luciferase reporter gene assay) allow one of ordinary skill to determine whether a sequence variant exhibits requisite cell status-specific transcription function. Hence, the invention also includes functionally-preserved variants of the nucleic acid sequences disclosed herein, which include nucleic acid substitutions, additions, and/or deletions. While not wishing to be bound by a single theory, the inventors note that it is possible that certain modifications will result in modulated resultant expression levels, including enhanced expression levels. Achievement of modulated resultant expression levels, preferably enhanced expression levels, may be especially desirable in the case of certain, more aggressive forms of cancer, or when a more rapid and/or aggressive pattern of cell killing is warranted (due to an immunocompromised condition of the individual, for example).

As an example of how cell status-specific TRE activity can be determined, a polynucleotide sequence or set of such sequences can be generated using methods known in the art, such as chemical synthesis, site-directed mutagenesis, PCR, and/or recombinant methods. The sequence(s) to be tested is inserted into a vector containing an appropriate reporter gene, including, but not limited to, chloramphenicol acetyl transferase (CAT), β -galactosidase (encoded by the lacZ gene), luciferase (encoded by the luc gene), green fluorescent protein, alkaline phosphatase, and horse radish peroxidase. Such vectors and assays are readily available, from, inter alia, commercial sources. Plasmids thus constructed

are transfected into a suitable host cell to test for expression of the reporter gene as controlled by the putative cell status-specific TRE using transfection methods known in the art, such as calcium phosphate precipitation, electroporation, liposomes (lipofection), and DEAE-dextran. Suitable host cells include any cell type, including but not limited to, Hep3B, Hep G2, HuH7, HuH1/C12, LNCaP, HBL-100, Chang liver cells, MCF-7, HLF, HLE, 3T3, HUVEC, and HeLa. Host cells transfected with the TRE-reporter gene construct to be tested are subjected to conditions which result in a change in cell status (for example, one which result in an aberrant physiological state). The same cells not subjected to these conditions, i.e., which are under normal physiological conditions and therefore in a normal physiological state, serve as controls. Results are obtained by measuring the level of expression of the reporter gene using standard assays. Comparison of expression between cells in a particular state and control indicates presence or absence of transcriptional activation. "Transcriptional activation" has been defined above.

Comparisons between or among various cell status-specific TREs can be assessed, for example, by measuring and comparing levels of expression within a single cell line under normal and aberrant physiological conditions. These comparisons may also be made by measuring and comparing levels of expression within a single cell line subjected to reversible environmental conditions (such as heat) and cells not subjected to such conditions. It is understood that absolute transcriptional activity of an cell status-specific TRE will depend on several factors, such as the nature of the target cell, delivery mode and form of the cell status-specific TRE, and the coding sequence that is to be selectively transcriptionally activated. To compensate for various plasmid sizes used, activities can be expressed as relative activity per mole of transfected plasmid. Alternatively, the level of transcription (i.e., mRNA) can be measured using standard Northern analysis and hybridization techniques. Levels of transfection (i.e., transfection efficiencies) are measured by co-transfecting a plasmid encoding a different reporter gene under control of a different TRE, such as the cytomegalovirus (CMV) immediate early promoter. This analysis can also indicate negative regulatory regions, i.e., silencers.

As an example of how hypoxia induction can be measured, one can use an assay such as that described in Jiang et al. (1997) *Cancer Research* 57:5328-5335 or Dachs et al. (1997)

Nature Med. 3:515-520. For example, a construct comprising a putative HRE, or multiple tandem copies thereof, together with a minimal promoter element, operably linked and controlling transcription of a polynucleotide which encodes a protein which is detectable or can be used to give a detectable signal, is introduced into host cells. The host cells are then subjected to conditions of normoxia (e.g., 20% O₂), and varying degrees of hypoxia, such as 5%, 2%, 1%, 0.1%, or less, O₂. The expression product of the operably linked polynucleotide (reporter gene) is then measured.

Alternatively a putative cell status-specific TRE can be assessed for its ability to confer adenoviral replication preference for cells exhibiting the requisite physiological state, such as heat or ionizing radiation. For this assay, constructs containing an adenovirus gene essential to replication operably linked to a putative cell status-specific TRE are transfected into cells which exhibit the requisite physiological state. Viral replication in those cells is compared, for example, to viral replication by the construct in cells under normal physiological conditions (i.e., not exhibiting the requisite physiological state).

Any of the various serotypes of adenovirus can be used, such as Ad2, Ad5, Ad12 and Ad40. For purposes of illustration, serotype Ad5 will be exemplified herein.

When a cell status-specific TRE is used with an adenovirus gene that is essential for propagation replication competence is preferentially achievable in the target cell expressing cell status. Preferably, the gene is an early gene, such as E1A, E1B, E2, or E4. (E3 is not essential for viral replication.) More preferably, the early gene under cell status-TRE control is E1A and/or E1B. More than one early gene can be placed under control of an cell status-specific TRE. Example 1 provides a more detailed description of such constructs.

The E1A gene is expressed immediately after viral infection (0-2 hours) and before any other viral genes. E1A protein acts as a *trans*-acting positive-acting transcriptional regulatory factor, and is required for the expression of the other early viral genes E1B, E2, E3, E4, and the promoter-proximal major late genes. Despite the nomenclature, the promoter proximal genes driven by the major late promoter are expressed during early times after Ad5 infection. Flint (1982) *Biochem. Biophys. Acta* 651:175-208; Flint (1986) *Advances Virus Research* 31:169-228; Grand (1987) *Biochem. J.* 241:25-38. In the absence of a functional E1A gene, viral infection does not proceed, because the gene products necessary for viral DNA

replication are not produced. Nevins (1989) *Adv. Virus Res.* 31:35–81. The transcription start site of Ad5 E1A is at 498 and the ATG start site of the E1A protein is at 560 in the virus genome.

5 The E1B protein functions *in trans* and is necessary for transport of late mRNA from the nucleus to the cytoplasm. Defects in E1B expression result in poor expression of late viral proteins and an inability to shut off host cell protein synthesis. The promoter of E1B has been implicated as the defining element of difference in the host range of Ad40 and Ad5: clinically Ad40 is an enterovirus, whereas Ad5 causes acute conjunctivitis. Bailey, Mackay et al. (1993) *Virology* 193:631; Bailey et al. (1994) *Virology* 202:695-706). The E1B promoter of Ad5
10 consists of a single high-affinity recognition site for Spl and a TATA box.

 The E2 region of adenovirus codes for proteins related to replication of the adenoviral genome, including the 72 kDa DNA-binding protein, the 80 kD precursor terminal protein and the viral DNA polymerase. The E2 region of Ad5 is transcribed in a rightward orientation from two promoters, termed E2 early and E2 late, mapping at 76.0 and 72.0 map units,
15 respectively. While the E2 late promoter is transiently active during late stages of infection and is independent of the E1A transactivator protein, the E2 early promoter is crucial during the early phases of viral replication.

 The E2 late promoter overlaps with the coding sequences of a gene encoded by the counterstrand and is therefore not amenable to genetic manipulation. However, the E2 early
20 promoter overlaps only for a few base pairs with sequences coding for a 33 kD protein on the counterstrand. Notably, the SpeI restriction site (Ad5 position 27082) is part of the stop codon for the above mentioned 33 kD protein and conveniently separates the major E2 early transcription initiation site and TATA-binding protein site from the upstream transcription factor binding sites E2F and ATF. Therefore, insertion of a cell status-TRE having SpeI ends
25 into the SpeI site in the +-strand would disrupt the endogenous E2 early promoter of Ad5 and should allow cell status-restricted expression of E2 transcripts.

 The E4 gene has a number of transcription products. The E4 region codes for two polypeptides which are responsible for stimulating the replication of viral genomic DNA and for stimulating late gene expression. The protein products of open reading frames (ORFS) 3
30 and 6 can both perform these functions by binding the 55kD protein from E1B and

heterodimers of E2F-1 and DP-1. The ORF 6 protein requires interaction with the E1B 55kD protein for activity while the ORF 3 protein does not. In the absence of functional protein from ORF 3 and ORF 6, plaques are produced with an efficiency less than 10^{-6} that of wild type virus. To further restrict viral replication to cells exhibiting a requisite physiological condition or state, E4 ORFs 1-3 can be deleted, making viral DNA replication and late gene synthesis dependent on E4 ORF 6 protein. By combining such a mutant with sequences in which the E1B region is regulated by a cell status-specific TRE, a virus can be obtained in which both the E1B function and E4 function are dependent on a cell status-specific TRE driving E1B.

The major late genes relevant to the subject invention are genes L1, L2, L3, L4, and L5 which encode proteins of the adenovirus virion. All of these genes (typically coding for structural proteins) are probably required for adenoviral replication. The late genes are all under the control of the major late promoter (MLP), which is located in Ad5 at +5986 to +6048.

In addition to conferring selective cytotoxic and/or cytolytic activity by virtue of preferential replication competence in cells exhibiting a requisite physiological state (for example, an aberrant physiological state such as low oxygen conditions), the adenovirus vectors of this invention can further include a heterologous gene (transgene) under the control of a cell status-specific TRE. In this way, various genetic capabilities may be introduced into target cells, particularly cancer cells. For example, in certain instances, it may be desirable to enhance the degree and/or rate of cytotoxic activity, due to, for example, the relatively refractory nature or particular aggressiveness of the cancerous target cell. This could be accomplished by coupling the cell status-specific replicative cytotoxic activity with cell-specific expression of, for example, HSV-tk and/or cytosine deaminase (cd), which renders cells capable of metabolizing 5-fluorocytosine (5-FC) to the chemotherapeutic agent 5-fluorouracil (5-FU). Using these types of transgenes may also confer a bystander effect.

Other desirable transgenes that may be introduced via an adenovirus vector(s) include genes encoding cytotoxic proteins, such as the A chains of diphtheria toxin, ricin or abrin (Palmiter et al. (1987) *Cell* 50: 435; Maxwell et al. (1987) *Mol. Cell. Biol.* 7: 1576; Behringer et al. (1988) *Genes Dev.* 2: 453; Messing et al. (1992) *Neuron* 8: 507; Piatak et al. (1988) *J.*

Biol. Chem. 263: 4937; Lamb et al. (1985) *Eur. J. Biochem.* 148: 265; Frankel et al. (1989) *Mol. Cell. Biol.* 9: 415), genes encoding a factor capable of initiating apoptosis, sequences encoding antisense transcripts or ribozymes, which among other capabilities may be directed to mRNAs encoding proteins essential for proliferation, such as structural proteins, or transcription factors; viral or other pathogenic proteins, where the pathogen proliferates intracellularly; genes that encode an engineered cytoplasmic variant of a nuclease (e.g. RNase A) or protease (e.g. aprotinin, papain, proteinase K, carboxypeptidase, etc.), or encode the Fas gene, and the like. Other genes of interest include cytokines, antigens, transmembrane proteins, and the like, such as IL-1, -2, -6, -12, GM-CSF, G-CSF, M-CSF, IFN- α , - β , - γ , TNF- α , - β , TGF- α , - β , NGF, and the like. The positive effector genes could be used in an earlier phase, followed by cytotoxic activity due to replication.

In one embodiment, the adenovirus death protein (ADP), encoded within the E3 region, is maintained in the adenovirus vector. The ADP gene, under control of the major late promoter (MLP), appears to code for a protein (ADP) that is important in expediting host cell lysis. Tollefson et al. (1996) *J. Virol.* 70(4):2296; Tollefson et al. (1992) *J. Virol.* 66(6):3633. Thus, adenoviral vectors containing the ADP gene may render the adenoviral vector more potent, making possible more effective treatment and/or a lower dosage requirement.

Accordingly, the invention provides an adenoviral vector as described herein that further includes a polynucleotide sequence encoding an ADP. A DNA sequence encoding an ADP and the amino acid sequence of an ADP are depicted Figure 9. Briefly, an ADP coding sequence is obtained preferably from Ad2 (since this is the strain in which ADP has been more fully characterized) using techniques known in the art, such as PCR. Preferably, the Y leader (which is an important sequence for correct expression of late genes) is also obtained and ligated to the ADP coding sequence. The ADP coding sequence (with or without the Y leader) can then be introduced into the adenoviral genome, for example, in the E3 region (where the ADP coding sequence will be driven by the MLP). The ADP coding sequence could also be inserted in other locations of the adenovirus genome, such as the E4 region. Alternatively, the ADP coding sequence could be operably linked to a heterologous promoter (with or without enhancer(s)), including, but not limited to, another viral promoter, a cell status-specific TRE

such as a hypoxia responsive element, or a cell type-specific TRE such as those derived from carcinoembryonic antigen (CEA), mucin, and rat probasin genes.

Adenoviral vectors of the invention further comprising a cell type specific element

5 In addition to conferring selective cytotoxic and/or cytolytic activity by virtue of preferential replication competence and/or by preferential transcription of a gene encoding a cytotoxic factor in cells exhibiting a requisite physiological state, the adenovirus vectors of this invention can further include an adenovirus gene and/or a heterologous gene (transgene) under the control of a cell type-specific TRE. In this way, cytotoxicity is further limited to a particular cell type.

10 For example, TREs that function preferentially in prostate cells include, but are not limited to, TREs derived from the prostate-specific antigen gene (*PSA*-TRE) (U.S. Patent No. 5,648,478), the glandular kallikrein-1 gene (from the human gene, *hKLK2*-TRE), and the probasin gene (*PB*-TRE) (International Patent Application No. PCT/US98/04132). All three of these genes are preferentially expressed in prostate cells and the expression is androgen-inducible. Generally, expression of genes responsive to androgen induction requires the
15 presence of an androgen receptor (AR).

PSA is synthesized exclusively by normal, hyperplastic, and malignant prostatic epithelia; hence, its tissue-specific expression has made it an excellent biomarker for benign prostatic hyperplasia (BPH) and prostatic carcinoma (CaP). Normal serum levels of PSA are
20 typically below 5 ng/ml, with elevated levels indicative of BPH or CaP. Lundwall et al. (1987) *FEBS Lett.* 214: 317; Lundwall (1989) *Biochem. Biophys. Res. Comm.* 161: 1151; and Riegmann et al. (1991) *Molec. Endocrin.* 5: 1921.

The region of the *PSA* gene that is used to provide cell specificity dependent upon androgens, particular in prostate cells, involves approximately 6.0 kilobases. Schuur et al.
25 (1996) *J. Biol. Chem.* 271:7043-7051. An enhancer region of approximately 1.5 kb in humans is located between nt -5322 and nt -3738, relative to the transcription start site of the *PSA* gene. The *PSA* promoter consists of the sequence from about nt -540 to nt +12 relative to the transcription start site. Juxtapositioning of these two genetic elements yield a fully functional, minimal prostate-specific enhancer/promoter (*PSE*) TRE. Other portions of the approximately

6.0 kb region of the *PSA* gene can be used in the present invention, as long as requisite functionality is maintained. In Example 1, adenoviral vector CN796 is described which comprises a composite TRE comprising an HRE and a PSA-TRE, the PSA-TRE comprising a PSA enhancer from -5322 to -3738 fused to a PSA promoter from -541 to +12. This PSA-TRE is derived from adenoviral vector CN706. Rodriguez et al. (1997) *Cancer Research* 57:2559-2563. Accordingly, in one embodiment an adenoviral vector comprises and adenovirus E1A gene under transcriptional control of a composite TRE comprising the cell status-specific TRE, HRE, and a cell type-specific TRE, a PSA-TRE.

The *PSE* and *PSA* TRE used in the present invention are derived from sequences depicted in Figure 4 (SEQ ID NO:3). The enhancer element is nucleotides about 503 to about 2086 of Figure 4 (SEQ ID NO:3). The promoter is nucleotides about 5285 to about 5836 of Figure 4 (SEQ ID NO:3). Accordingly, in some embodiments, the composite TRE comprises an HRE comprising SEQ ID NO:1 and a PSA-TRE comprises nucleotides about 503 to about 2086 of SEQ ID NO:3. In other embodiments, the composite TRE comprises an HRE comprising SEQ ID NO:1 and a PSA-TRE comprises nucleotides about 503 to about 2086 of SEQ ID NO:3 and nucleotides about 5285 to about 5836 of SEQ ID NO:3. As described above, these composite (HRE/PSA) TREs may be operably linked to an adenovirus gene essential for replication, especially an early gene such as E1A or E1B. Example 1 describes such a construct.

In the present invention, replication-competent adenovirus vectors comprising a cell status-specific TRE and a cell type-specific TRE may employ cell type-specific TREs that are preferentially functional in particular tumor cells. Non-limiting examples of tumor cell-specific TREs, and non-limiting examples of respective potential target cells, include TREs from the following genes: α -fetoprotein (*AFP*) (liver cancer), mucin-like glycoprotein DF3 (*MUC1*) (breast carcinoma), carcinoembryonic antigen (*CEA*) (colorectal, gastric, pancreatic, breast, and lung cancers), plasminogen activator urokinase (*uPA*) and its receptor gene (breast, colon, and liver cancers), *HER-2/neu* (*c-erbB2/neu*) (breast, ovarian, stomach, and lung cancers).

Other cell type-specific TREs may be derived from the following exemplary genes (cell type in which the TREs are specifically functional are in parentheses): vascular endothelial growth factor receptor (endothelium), albumin (liver), factor VII (liver), fatty acid synthase (liver), von Willebrand factor (brain endothelium), alpha-actin and myosin heavy chain (both in smooth muscle), synthetase I (small intestine), Na-K-Cl transporter (kidney). Additional cell type-specific TREs are known in the art.

AFP is an oncofetal protein, the expression of which is primarily restricted to developing tissues of endodermal origin (yolk sac, fetal liver, and gut), although the level of its expression varies greatly depending on the tissue and the developmental stage. AFP is of clinical interest because the serum concentration of AFP is elevated in a majority of hepatoma patients, with high levels of AFP found in patients with advanced disease. The serum AFP levels in patients appear to be regulated by AFP expression in hepatocellular carcinoma but not in surrounding normal liver. Thus, the AFP gene appears to be regulated to hepatoma cell-specific expression.

Cell type-specific TREs from the *AFP* gene have been identified. For example, the cloning and characterization of human AFP-specific enhancer activity is described in Watanabe et al. (1987) *J. Biol. Chem.* 262:4812-4818. The entire 5' *AFP* flanking region (containing the promoter, putative silencer, and enhancer elements) is contained within approximately 5 kb upstream from the transcription start site.

The *AFP* enhancer region in human is located between about nt -3954 and about nt -3335, relative to the transcription start site of the *AFP* gene. The human *AFP* promoter encompasses a region from about nt -174 to about nt +29. Juxtapositioning of these two genetic elements yields a fully functional *AFP*-TRE. Ido et al. (1995) describe a 259 bp promoter fragment (nt -230 to nt +29) that is specific for HCC. *Cancer Res.* 55:3105-3109. The *AFP* enhancer contains two regions, denoted A and B, located between nt -3954 and nt -3335 relative to the transcription start site. The promoter region contains typical TATA and CAAT boxes. Preferably, the *AFP*-TRE contains at least one enhancer region. More preferably, the *AFP*-TRE contains both enhancer regions.

Suitable target cells for adenoviral vectors containing *AFP*-TREs are any cell type that allow an *AFP*-TRE to function. Preferred are cells that express, or produce, AFP, including,

but not limited to, tumor cells expressing AFP. Examples of such cells are hepatocellular carcinoma cells, gonadal and other germ cell tumors (especially endodermal sinus tumors), brain tumor cells, ovarian tumor cells, acinar cell carcinoma of the pancreas (Kawamoto et al. (1992) *Hepatogastroenterology* 39:282-286), primary gall bladder tumor (Katsuragi et al. (1989) *Rinsko Hoshasen* 34:371-374), uterine endometrial adenocarcinoma cells (Koyama et al. (1996) *Jpn. J. Cancer Res.* 87:612-617), and any metastases of the foregoing (which can occur in lung, adrenal gland, bone marrow, and/or spleen). In some cases, metastatic disease to the liver from certain pancreatic and stomach cancers produce AFP. Especially preferred are hepatocellular carcinoma cells and any of their metastases. AFP production can be measured using assays standard in the art, such as RIA, ELISA or Western blots (immunoassays) to determine levels of AFP protein production or Northern blots to determine levels of AFP mRNA production. Alternatively, such cells can be identified and/or characterized by their ability to activate transcriptionally an *AFP-TRE* (i.e., allow an *AFP-TRE* to function).

The protein urokinase plasminogen activator (uPA) and its cell surface receptor, urokinase plasminogen activator receptor (uPAR), are expressed in many of the most frequently occurring neoplasia and appear to represent important proteins in cancer metastasis. Both proteins are implicated in breast, colon, prostate, liver, renal, lung and ovarian cancer. Transcriptional regulatory elements that regulate uPA and uPAR transcription have been extensively studied. Riccio et al. (1985) *Nucleic Acids Res.* 13:2759-2771; Cannio et al. (1991) *Nucleic Acids Res.* 19:2303-2308.

CEA is a 180,000-Dalton glycoprotein tumor-associated antigen present on endodermally-derived neoplasia of the gastrointestinal tract, such as colorectal, gastric (stomach) and pancreatic cancer, as well as other adenocarcinomas such as breast and lung cancers. CEA is of clinical interest because circulating CEA can be detected in the great majority of patients with CEA-positive tumors. In lung cancer, about 50% of total cases have circulating CEA, with high concentrations of CEA (greater than 20 ng/ml) often detected in adenocarcinomas. Approximately 50% of patients with gastric carcinoma are serologically positive for CEA.

The 5' upstream flanking sequence of the *CEA* gene has been shown to confer cell-specific activity. The *CEA* promoter region, approximately the first 424 nucleotides upstream of the translational start site in the 5' flanking region of the gene, was shown to confer cell-specific activity when the region provided higher promoter activity in *CEA*-producing cells than in non-producing HeLa cells.. Schrewe et al. (1990) *Mol. Cell. Biol.* 10:2738-2748. In addition, cell-specific enhancer regions have been found. WO/95/14100. The entire 5' *CEA* flanking region (containing the promoter, putative silencer, and enhancer elements) appears to be contained within approximately 14.5 kb upstream from the transcription start site. Richards et al. (1995); WO 95/14100. Further characterization of the 5' flanking region of the *CEA* gene by Richards et al. (1995) indicated two upstream regions, -13.6 to -10.7 kb or -6.1 to -4.0 kb, when linked to the multimerized promoter resulted in high-level and selective expression of a reporter construct in *CEA*-producing LoVo and SW1463 cells. Richards et al. (1995) also localized the promoter region to nt -90 and nt +69 relative to the transcriptional start site, with region nt -41 to nt -18 as essential for expression. WO95/14100 describes a series of 5' flanking *CEA* fragments which confer cell-specific activity, such as about nt -299 to about nt +69; about nt -90 to about nt +69; nt -14,500 to nt -10,600; nt -13,600 to nt -10,600, nt -6100 to nt -3800. In addition, cell specific transcription activity is conferred on an operably linked gene by the *CEA* fragment from nt -402 to nt +69, depicted in (SEQ ID NO:6). Any *CEA*-TREs used in the present invention are derived from mammalian cells, including but not limited to, human cells. Thus, any of the *CEA*-TREs may be used in the invention as long as requisite desired functionality is displayed in the adenovirus vector. The cloning and characterization of *CEA* sequences have been described in the literature and are thus made available for practice of this invention and need not be described in detail herein.

The protein product of the *MUC1* gene (known as mucin or MUC1 protein; episialin; polymorphic epithelial mucin or PEM; EMA; DF3 antigen; NPGP; PAS-O; or CA15.3 antigen) is normally expressed mainly at the apical surface of epithelial cells lining the glands or ducts of the stomach, pancreas, lungs, trachea, kidney, uterus, salivary glands, and mammary glands. Zotter et al. (1988) *Cancer Rev.* 11-12: 55-101; and Girling et al. (1989) *Int. J. Cancer* 43: 1072-1076. However, mucin is overexpressed in 75-90% of human breast carcinomas. Kufe et al. (1984) *Hybridoma* 3: 223-232. For reviews, see Hilkens (1988)

Cancer Rev. 11-12: 25-54; and Taylor-Papadimitriou, et al. (1990) *J. Nucl. Med. Allied Sci.* 34: 144-150. Mucin protein expression correlates with the degree of breast tumor differentiation. Lundy et al. (1985) *Breast Cancer Res. Treat.* 5: 269-276. This overexpression appears to be controlled at the transcriptional level.

5 Overexpression of the *MUC1* gene in human breast carcinoma cells MCF-7 and ZR-75-1 appears to be regulated at the transcriptional level. Kufe et al. (1984); Kovarik (1993) *J. Biol. Chem.* 268:9917-9926; and Abe et al. (1990) *J. Cell. Physiol.* 143: 226-231. The regulatory sequences of the *MUC1* gene have been cloned, including the approximately 0.9 kb upstream of the transcription start site which contains a TRE that appears to be involved in
10 cell-specific transcription. Abe et al. (1993) *Proc. Natl. Acad. Sci. USA* 90: 282-286; Kovarik et al. (1993); and Kovarik et al. (1996) *J. Biol. Chem.* 271:18140-18147.

Any *MUC1*-TREs used in the present invention are derived from mammalian cells, including but not limited to, human cells. Preferably, the *MUC1*-TRE is human. In one embodiment, the *MUC1*-TRE may contain the entire 0.9 kb 5' flanking sequence of the *MUC1*
15 gene. In other embodiments, the *MUC1*-TREs comprise the following sequences (relative to the transcription start site of the *MUC1* gene): about nt -725 to about nt +31, nt -743 to about nt +33, nt -750 to about nt +33, and nt -598 to about nt +485 (operably-linked to a promoter).

The *c-erbB2/neu* gene (*HER-2/neu* or *HER*) is a transforming gene that encodes a 185 kD epidermal growth factor receptor-related transmembrane glycoprotein. In humans, the *c-erbB2/neu* protein is expressed during fetal development, however, in adults, the protein is
20 weakly detectable (by immunohistochemistry) in the epithelium of many normal tissues. Amplification and/or over-expression of the *c-erbB2/neu* gene has been associated with many human cancers, including breast, ovarian, uterine, prostate, stomach and lung cancers. The clinical consequences of the *c-erbB2/neu* protein over-expression have been best studied in
25 breast and ovarian cancer. *c-erbB2/neu* protein over-expression occurs in 20 to 40% of intraductal carcinomas of the breast and 30% of ovarian cancers, and is associated with a poor prognosis in subcategories of both diseases. Human, rat and mouse *c-erbB2/neu* TREs have been identified and shown to confer *c-erbB2/neu* expressing cell specific activity. Tal et al. (1987) *Mol. Cell. Biol.* 7:2597-2601; Hudson et al. (1990) *J. Biol. Chem.* 265:4389-4393;

Grooteclaes et al. (1994) *Cancer Res.* 54:4193–4199; Ishii et al. (1987) *Proc. Natl. Acad. Sci. USA* 84:4374–4378; Scott et al. (1994) *J. Biol. Chem.* 269:19848–19858.

5 The cell type-specific TREs listed above are provided as non-limiting examples of TREs that would function in the instant invention. Additional cell-specific TREs are known in the art, as are methods to identify and test cell specificity of suspected TREs.

Using the adenoviral vectors of the invention

10 The adenoviral vectors can be used in a variety of forms, including, but not limited to, naked polynucleotide (usually DNA) constructs; polynucleotide constructs complexed with agents to facilitate entry into cells, such as cationic liposomes or other cationic compounds such as polylysine; packaged into infectious adenovirus particles (which may render the
15 adenoviral vector(s) more immunogenic); packaged into other particulate viral forms such as HSV or AAV; complexed with agents (such as PEG) to enhance or dampen an immune response; complexed with agents that facilitate *in vivo* transfection, such as DOTMATM, DOTAPTM, and polyamines. Thus, the invention also provides an adenovirus capable of replicating preferentially in cell status-producing cells. “Replicating preferentially” means that
20 the adenovirus replicates more in cell exhibiting a requisite physiological state than a cell not exhibiting that state. Preferably, the adenovirus replicates at least about 2-fold higher, preferably at least about 5-fold higher, more preferably at least about 10-fold higher, still more preferably at least about 50-fold higher, even more preferably at least about 100-fold higher, still more preferably at least about 400-fold to about 500-fold higher, still more preferably at least about 1000-fold higher, most preferably at least about 1×10^6 higher. Most preferably, the adenovirus replicates solely in cells exhibiting a requisite physiological state (that is, does not replicate or replicates at very low levels in cells not exhibiting the requisite physiological state).

25 If an adenoviral vector is packaged into an adenovirus, the adenovirus itself may also be selected to further enhance targeting. For example, adenovirus fibers mediate primary contact with cellular receptor(s) aiding in tropism. See, e.g., Amberg et al. (1997) *Virology* 227:239-244. If a particular subgenus of an adenovirus serotype displayed tropism for a target

cell type and/or reduced affinity for non-target cell types, such subgenus (or subgenera) could be used to further increase cell-specificity of cytotoxicity and/or cytolysis.

The adenoviral vectors may be delivered to the target cell in a variety of ways, including, but not limited to, liposomes, general transfection methods that are well known in the art (such as calcium phosphate precipitation or electroporation), direct injection, and intravenous infusion. The means of delivery will depend in large part on the particular adenoviral vector (including its form) as well as the type and location of the target cells (i.e., whether the cells are *in vitro* or *in vivo*).

If used as a packaged adenovirus, adenovirus vectors may be administered in an appropriate physiologically acceptable carrier at a dose of about 10^4 to about 10^{14} . The multiplicity of infection will generally be in the range of about 0.001 to 100. If administered as a polynucleotide construct (i.e., not packaged as a virus) about 0.01 μg to about 1000 μg of an adenoviral vector can be administered. The adenoviral vector(s) may be administered one or more times, depending upon the intended use and the immune response potential of the host, and may also be administered as multiple, simultaneous injections. If an immune response is undesirable, the immune response may be diminished by employing a variety of immunosuppressants, so as to permit repetitive administration, without a strong immune response. If packaged as another viral form, such as HSV, an amount to be administered is based on standard knowledge about that particular virus (which is readily obtainable from, for example, published literature) and can be determined empirically.

Host cells comprising the adenoviral vectors of the invention

The present invention also provides host cells comprising (i.e., transformed with) the adenoviral vectors described herein. Both prokaryotic and eukaryotic host cells can be used as long as sequences requisite for maintenance in that host, such as appropriate replication origin(s), are present. For convenience, selectable markers are also provided. Prokaryotic host cells include bacterial cells, for example, *E. coli* and mycobacteria. Among eukaryotic host cells are yeast, insect, avian, plant and mammalian. Host systems are known in the art and need not be described in detail herein.

Compositions of the invention

The present invention also provides compositions, including pharmaceutical compositions, containing the adenoviral vectors described herein. Such compositions (especially pharmaceutical compositions) are useful for administration *in vivo*, for example, when measuring the degree of transduction and/or effectiveness of cell killing in an individual. Pharmaceutical compositions, comprised an adenoviral vector of this invention in a pharmaceutically acceptable excipient (generally an effective amount of the adenoviral vector), are suitable for systemic administration to individuals in unit dosage forms, sterile parenteral solutions or suspensions, sterile non-parenteral solutions or oral solutions or suspensions, oil in water or water in oil emulsions and the like. Formulations for parenteral and nonparenteral drug delivery are known in the art and are set forth in *Remington's Pharmaceutical Sciences*, 19th Edition, Mack Publishing (1995). Pharmaceutical compositions also include lyophilized and/or reconstituted forms of the adenoviral vectors (including those packaged as a virus, such as adenovirus) of the invention.

Other compositions are used, and are useful for, detection methods described herein. For these compositions, the adenoviral vector usually is suspended in an appropriate solvent or solution, such as a buffer system. Such solvent systems are well known in the art.

Kits of the invention

The present invention also encompasses kits containing an adenoviral vector(s) of this invention. These kits can be used for diagnostic and/or monitoring purposes, preferably monitoring. Procedures using these kits can be performed by clinical laboratories, experimental laboratories, medical practitioners, or private individuals. Kits embodied by this invention allow someone to detect the presence of cell status-producing cells in a suitable biological sample, such as biopsy specimens.

The kits of the invention comprise an adenoviral vector described herein in suitable packaging. The kit may optionally provide additional components that are useful in the procedure, including, but not limited to, buffers, developing reagents, labels, reacting surfaces, means for detection, control samples, instructions, and interpretive information.

Preparation of the adenovirus vectors of the invention

The adenovirus vectors of this invention can be prepared using recombinant techniques that are standard in the art. Generally, a cell status-specific TRE is inserted 5' to the adenoviral gene of interest, preferably one or more early genes (although late gene(s) may be used). A cell status-specific TRE can be prepared using oligonucleotide synthesis (if the sequence is known) or recombinant methods (such as PCR and/or restriction enzymes). Convenient restriction sites, either in the natural adeno-DNA sequence or introduced by methods such as oligonucleotide directed mutagenesis and PCR, provide an insertion site for a cell status-specific TRE. Accordingly, convenient restriction sites for annealing (i.e., inserting) a cell status-specific TRE can be engineered onto the 5' and 3' ends of a cell status-specific TRE using standard recombinant methods, such as PCR.

Polynucleotides used for making adenoviral vectors of this invention may be obtained using standard methods in the art, such as chemical synthesis, by recombinant methods, and/or by obtaining the desired sequence(s) from biological sources.

Adenoviral vectors are conveniently prepared by employing two plasmids, one plasmid providing for the left hand region of adenovirus and the other plasmid providing for the right hand region, where the two plasmids share at least about 500 nt of middle region for homologous recombination. In this way, each plasmid, as desired, may be independently manipulated, followed by cotransfection in a competent host, providing complementing genes as appropriate, or the appropriate transcription factors for initiation of transcription from a cell status-specific TRE for propagation of the adenovirus. Plasmids are generally introduced into a suitable host cell such as 293 cells using appropriate means of transduction, such as cationic liposomes. Alternatively, *in vitro* ligation of the right and left-hand portions of the adenovirus genome can also be used to construct recombinant adenovirus derivative containing all the replication-essential portions of adenovirus genome. Berkner et al. (1983) *Nucleic Acid Research* 11: 6003-6020; Bridge et al. (1989) *J. Virol.* 63: 631-638.

For convenience, plasmids are available that provide the necessary portions of adenovirus. Plasmid pXC.1 (McKinnon (1982) *Gene* 19:33-42) contains the wild-type left-hand end of Ad5. pBHG10 (Bett et al. (1994) *Proc. Natl. Acad. Sci USA* 91:8802-8806; Microbix Biosystems Inc., Toronto) provides the right-hand end of Ad5, with a deletion in E3.

The deletion in E3 provides room in the virus to insert a 3 kb cell status-TRE without deleting the endogenous enhancer/promoter. Bett et al. (1994). The gene for E3 is located on the opposite strand from E4 (r-strand). pBHG11 provides an even larger E3 deletion (an additional 0.3 kb is deleted). Bett et al. (1994).

5 For manipulation of the early genes, the transcription start site of Ad5 E1A is at 498 and the ATG start site of the E1A protein is at 560 in the virus genome. This region can be used for insertion of an cell status-specific TRE. A restriction site may be introduced by employing polymerase chain reaction (PCR), where the primer that is employed may be limited to the Ad5 genome, or may involve a portion of the plasmid carrying the Ad5 genomic
10 DNA. For example, where pBR322 is used, the primers may use the EcoRI site in the pBR322 backbone and the XbaI site at 1339 of Ad5. By carrying out the PCR in two steps, where overlapping primers at the center of the region introduce a 30 sequence change resulting in a unique restriction site, one can provide for insertion of heterologous TRE at that site.

 A similar strategy may also be used for insertion of a heterologous TRE to regulate
15 E1B. The E1B promoter of Ad5 consists of a single high-affinity recognition site for Spl and a TATA box. This region extends from 1636 to 1701. By insertion of a heterologous TRE in this region, one can provide for target cell-specific transcription of the E1B gene. By employing the left-hand region modified with a heterologous TRE regulating E1A as the template for introducing a heterologous TRE to regulate E1B, the resulting adenovirus vector
20 will be dependent upon the cell status-specific transcription factors for expression of both E1A and E1B.

 Similarly, a cell status-specific TRE can be inserted upstream of the E2 gene to make its expression cell status specific. The E2 early promoter, mapping in Ad5 from 27050-27150, consists of a major and a minor transcription initiation site, the latter accounting for about 5%
25 of the E2 transcripts, two non-canonical TATA boxes, two E2F transcription factor binding sites and an ATF transcription factor binding site. For a detailed review of the E2 promoter architecture see Swaminathan et al., *Curr. Topics in Micro. and Imm.* (1995) 199 (part 3):177-194.

 For E4, one must use the right hand portion of the adenovirus genome. The E4
30 transcription start site is predominantly at 35609, the TATA box at 35638 and the first

ATG/CTG of ORF 1 is at 35532. Virtanen et al. (1984) *J. Virol.* 51: 822-831. Using any of the above strategies for the other genes, a cell status-specific TRE may be introduced upstream from the transcription start site. For the construction of mutants in the E4 region, the co-transfection and homologous recombination are performed in W162 cells (Weinberg et al. (1983) *Proc. Natl. Acad. Sci.* 80:5383-5386) which provide E4 proteins *in trans* to complement defects in synthesis of these proteins. Alternatively, these constructs can be produced by *in vitro* ligation.

Methods using the adenovirus vectors of the invention

The adenoviral vectors of the invention can be used for a wide variety of purposes, which will vary with the desired or intended result. Accordingly, the present invention includes methods using the adenoviral vectors described above.

In one embodiment, methods are provided for conferring selective cytotoxicity in target cells (i.e., cells exhibiting a requisite physiological state which allows a cell status-specific TRE to function), generally but not necessarily in an individual (preferably human), comprising contacting the cells with an adenovirus vector described herein, such that the adenovirus vector enters the cell. Cytotoxicity can be measured using standard assays in the art, such as dye exclusion, ³H-thymidine incorporation, and/or lysis.

In another embodiment, methods are provided for propagating an adenovirus specific for mammalian cells which allow a cell status-specific TRE to function. These methods entail combining an adenovirus vector with mammalian cells, whereby said adenovirus is propagated.

The invention further provides methods of suppressing tumor cell growth, generally but not necessarily in an individual (preferably human), comprising contacting a tumor cell with an adenoviral vector of the invention such that the adenoviral vector enters the tumor cell and exhibits selective cytotoxicity for the tumor cell. Tumor cell growth can be assessed by any means known in the art, including, but not limited to, measuring tumor size, determining whether tumor cells are proliferating using a ³H-thymidine incorporation assay, or counting tumor cells.

The invention also includes methods for detecting target cells (i.e., cells which permit or induce a cell status-specific TRE to function) in a biological sample. These methods are particularly useful for monitoring the clinical and/or physiological condition of an individual (i.e., mammal), whether in an experimental or clinical setting. For these methods, cells of a biological sample are contacted with an adenovirus vector, and replication of the adenoviral vector is detected. A suitable biological sample is one in which cells exhibiting a requisite physiological (and/or environmental) state, for example, an aberrant physiological state (such as cells in hypoxic conditions and exhibiting a phenotype characteristic of cells in hypoxic conditions, such as expression of HIF-1) may be or are suspected to be present. Generally, in mammals, a suitable clinical sample is one in which cancerous cells exhibiting a requisite physiological state, such as cells within a solid tumor which are under hypoxic conditions, are suspected to be present. Such cells can be obtained, for example, by needle biopsy or other surgical procedure. Cells to be contacted may be treated to promote assay conditions, such as selective enrichment, and/or solubilization. In these methods, target cells can be detected using *in vitro* assays that detect adenoviral proliferation, which are standard in the art. Examples of such standard assays include, but are not limited to, burst assays (which measure virus yield) and plaque assays (which measure infectious particles per cell). Propagation can also be detected by measuring specific adenoviral DNA replication, which are also standard assays.

The following examples are provided to illustrate but not limit the invention.

EXAMPLES

EXAMPLE 1

Adenovirus vector comprising E1A under transcriptional control of a hypoxia responsive element and a PSA-TRE

General techniques

A human embryonic kidney cell line, 293, efficiently expresses E1A and E1B genes of Ad5 and exhibits a high transfection efficiency with adenovirus DNA. To generate

recombinant adenovirus, 293 cells were co-transfected with one left end Ad5 plasmid and one right end Ad5 plasmid. Homologous recombination generates adenoviruses with the required genetic elements for replication in 293 cells which provide E1A and E1B proteins *in trans* to complement defects in synthesis of these proteins.

5 The plasmids to be combined were co-transfected into 293 cells using cationic liposomes such as Lipofectin (DOTMA:DOPE™, Life Technologies) by combining the two plasmids, then mixing the plasmid DNA solution (10 µg of each plasmid in 500 µl of minimum essential medium (MEM) without serum or other additives) with a four-fold molar excess of liposomes in 200 µl of the same buffer. The DNA-lipid complexes were then placed
10 on the cells and incubated at 37°C, 5% CO₂ for 16 hours. After incubation the medium was changed to MEM with 10% fetal bovine serum and the cells are further incubated at 37°C, 5% CO₂, for 10 days with two changes of medium. At the end of this time the cells and medium were transferred to tubes, freeze-thawed three times, and the lysate was used to infect 293 cells at the proper dilution to detect individual viruses as plaques.

15 Plaques obtained were plaque purified twice, and viruses were characterized for presence of desired sequences by PCR and occasionally by DNA sequencing. For further experimentation, the viruses were purified on a large scale by cesium chloride gradient centrifugation.

Adenovirus vectors in which E1A is under transcriptional control of a cell status-specific TRE

20 An adenovirus vector containing a hypoxia response element (HRE) was generated. CN796, an adenovirus vector in which E1A is under the control of a composite TRE consisting of an HRE and a PSA-TRE, was made by co-transfecting CN515 with pBHG10. CN515 was constructed by inserting a 67 base pair fragment from HRE eno1 (Jiang et al. (1997) *Cancer Research* 57:5328-5335) (SEQ ID NO:1; Figure 2) into CN65 at the BglII site. CN65 is a
25 plasmid containing an enhancer and promoter from the human PSA gene, consisting of an enhancer from -5322 to -3738 fused to a PSA promoter from -541 to +12. This is the PSA-TRE contained within plasmid CN706. Rodriguez et al. (1997) *Cancer Res.* 57:2559-2563.

Virus growth in vitro

5 Growth selectivity of recombinant adenovirus is analyzed in plaque assays in which a single infectious particle produces a visible plaque by multiple rounds of infection and replication. Virus stocks are diluted to equal pfu/ml, then used to infect monolayers of cells for 1 hour. The inoculum is then removed and the cells are overlayed with semisolid agar containing medium and incubated at 37°C for 10 days. Plaques in the monolayer are then counted and titers of infectious virus on the various cells are calculated. The data are normalized to the titer of CN702 (wild type) on 293 cells.

Claims

What is claimed is:

1. An adenovirus vector comprising an adenovirus gene under transcriptional control of a transcriptional regulatory element (TRE) comprising a cell status-specific TRE.
- 5 2. The adenovirus vector of claim 1, wherein the adenovirus gene is essential for viral replication.
3. The adenovirus vector of claim 2, wherein the adenovirus gene is an early gene.
4. The adenovirus vector of claim 2, wherein the adenovirus gene is a late gene.
5. The adenovirus vector of claim 3, wherein the adenovirus early gene is E1A.
- 10 6. The adenovirus vector of claim 3, wherein the adenovirus early gene is E1B.
7. The adenovirus vector of claim 3, wherein the adenovirus early gene is E4.
8. The adenovirus vector of claim 1, wherein the cell status-specific TRE is human.
9. The adenovirus vector of claim 1, wherein the cell status-specific TRE comprises a hypoxia responsive element (HRE).
- 15 10. The adenovirus vector of claim 9, wherein the HRE comprises SEQ ID NO:1.
11. The adenovirus vector of claim 1, wherein the cell status-specific TRE comprises a cell cycle specific element.
12. The adenovirus vector of claim 11, wherein the cell cycle-specific element is from the E2F-1 gene.
- 20 13. The adenovirus vector of claim 1, wherein the cell status-specific TRE comprises a heat-inducible element.

14. The adenovirus vector of claim 1, further comprising a cell type-specific TRE.
15. The adenovirus vector of claim 14, wherein the cell type-specific TRE is prostate cell specific.
- 5 16. The adenovirus vector of claim 15, wherein the prostate cell-specific TRE is a *PSA*-TRE.
17. The adenovirus vector of claim 1, further comprising a transgene under transcriptional control of a second cell status-specific TRE.
18. An adenovirus vector comprising an adenovirus gene under transcriptional control of a TRE comprising a cell status-specific TRE and a cell-type specific TRE.
- 10 19. The adenovirus vector of claim 18, wherein the adenovirus gene is an early gene.
20. The adenovirus vector of claim 19, wherein the adenovirus early gene is E1A.
21. The adenovirus vector of claim 20, wherein the cell status-specific TRE comprises an HRE and the cell-type specific TRE is a *PSA*-TRE.
- 15 22. The adenovirus vector of claim 21, wherein the HRE comprises SEQ ID NO:1 and the *PSA*-TRE comprises nucleotides about 503 to about 2086 of SEQ ID NO:3 and nucleotides about 5285 to about 5836 of SEQ ID NO:3.
23. A composition comprising an adenovirus vector of claim 1.
24. The composition of claim 23, further comprising a pharmaceutically acceptable excipient.
- 20 25. A host cell comprising the adenovirus vector of claim 1.
26. A method of propagating adenovirus specific for cells which allow a cell status-specific TRE to function, said method comprising combining an adenovirus according to claim 1 with the cells, whereby said adenovirus is propagated.

27. A method for conferring selective cytotoxicity on a target cell, said method comprising contacting a cell which allows a cell status-specific TRE to function with an adenovirus vector of claim 1, whereby the vector enters the cell.

5 28. A method for suppressing tumor growth comprising introducing the adenovirus vector of claim 1 into a tumor cell which allows a cell status-specific TRE to function, wherein introduction of the adenovirus vector results in suppression of tumor growth.

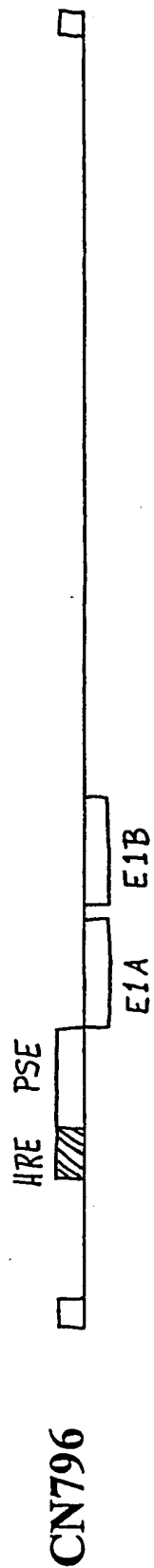


FIGURE 1

FIGURE 2

ccccgagg cagtgcac gaggtcagg gcgtgcgt ggtcgcagcagagaccccg ggtgcag gccgga

FIGURE 3

```

gggcccaaaa ttagcaagt accacgtggt tctgaagcca gtggcctaag gaccacctt 61
gcagaaccgt ggtctccttg tcacagtcta ggcagcctct ggcttagcct ctgtttcttt 121
cataaccttt ctacagcgctt gctctgggccc gcagatggag aagaccagtgt tgggaggagt cgctactgag 181
ctcctagatt ggcagggggag gaggaaagtga gaaatcctt ggtgtggtca gcatggagc 241
agaggcagca gtgggcaata gggaaagtga gaaatcctt gggagggtc ctagaagtg 301
atgtgttttc tttttttgtt ttagagacag gatctcgtc tgcgtccag gctgggtgtgc 361
agtggcatga tcatagctca ctgcagcctc gacttctcgg gctcaagcaa tctccacc 421
tcagcctccc aagtagctgg gactacgggc acagccacc atgcctggct aatttttgtta 481
ttttttgtag agatgggtct tcaccatgtt gatcaggctg gtctcgaact cctgggtca 541
tgcgatccac cccgccagct gattacaggg attccggtgg tgagccaccg cgccagacg 601
ccacttcac gtattgtaaa gctctgttac ctttctgttc cctgtctac tggactgtga 661
gtcccttagg gccacgaatt gaggatgggg cacagagcaa gctctccaaa cgtttgttga 721
atgagttagg gaatgaatga gttcaagcag atgctatag ttggctgttg gagattttgg 781
ctaaaaatggg acttgcaagg aagcccgacg tcccctcgc cattccagg caccgtctt 841
cagcttgggc tctgggtgag agaggtaggg ctgggtgcag gattaggata atgtcatggg 901
tgaggcaagt tgaggatgga agaggtggct gatggctggg ctgtggaact gatgatcctg 961
aaaagaagag gggacagtct ctggaatctt aagctgaggg tgttggggc tacaggttga 1021
gggtcacgtg cagaagagag gctctgttct gacctgcac tatagaaagg tcagtgggat 1081
gcgggagcgt cggggcgggg gggggcctat gttcccgtgt cccacgcct ccagcagggg 1141
acgccccggc tggggcgggg ggtcagacc gcgctggta ccattccggac aaagcctgcg 1201
cgcccccgcc cccgccattg gccgtaccgc cccgcgcgc cgcctccgc cgcccccg 1261
cgccccggcc ggcgcgttaa agccaatagg aaccgcgcg gttgttccg tcacggccgg 1321
ggcagccaat tttggcgggc ctcggcggtc cgtggctctt tcgctggcaa aagatttg 1381
cgcgtaaaag* tggccgggac ttgcaaggc gcggcgggc ggggcggagc gggatcgagc 1441
cctcgccgag gcctgccgc atgggccccg gccgcgcgc cgcctgtca cccggggccg 1501
gcgggcccgtg agcgtcatg

```

FIGURE 4A

aagcttctag ttttcttttc ccggtgacat cgtggaaagc actagcatct ctaagcaatg 60
atctgtgaca atattcacag tgtaatgcca tccagggaac tcaactgagc cttgatgtcc 120
agagattttt gtgttttttt ctgagactga gtctcgctct gtgccaggct ggagtgcagt 180
ggtgcaacct tggtcactg caagctccgc ctcttggtt cagccattc tcctgcctca 240
gcctcctgag tagctgggac tacaggcacc cgccaccacg cctggctaata tttttgtat 300
ttttagtaga gatggggttt cactgtgtta gccaggatgg tctcagctct ctgacctcgt 360
gatctgccc ccttgccctc ccaaagtgt gggatgacag gcgtgagcca ccgcgcctgg 420
ccgatatcca gagatttttt ggggggctcc atcacacaga catgttgac gtcttcattg 480
ttgactttta gtatccagcc cctctagaaa tctagctgat atagtgtggc tcaaaactt 540
cagcacaat cacaccgtta gactatctgg tgtggcccaa accttcagg gaacaaagg 600
actetaatct ggcaggatat tccaaagcat tagagatgac ctcttgcaa gaaaaagaa 660
tggaagaa aaagaaagaa aggaagaaaa aaaaaaaa gagatgacct ctgaggtct 720
gaggggaaac gcctgaggtc tttgagcaag gtcagtcctc tgttgacag tctccctcac 780
agggtcattg tgacgatcaa atgtgtcac gtgtatgagg caccagcaca tgcttggtc 840
tggggagtgc cgtgtaagt tatgcttgca ctgctgaatg cttgggatgt gtcagggtt 900
atcttcagca cttacagatg ctcatctcat cctcacagca tcaactatgg atgggtatta 960
ctggcctcat ttgatggaga aagtggctgt ggctcagaaa ggggggacca ctagaccagg 1020
gacactctgg atgtgggga ctccagagac catgaccact caccaactgc agagaaatta 1080
attgtggcct gatgtccctg tcctggagag ggtggagggtg gaccttact aacctctac 1140
cttgaccctc tcttttaggg ctctttctga cctccaccat ggtactagga cccattgta 1200
ttctgtacc tctgactct atgaccccca ctgcccactg catccagctg ggtccctcc 1260
tatctctatt ccagctggc cagtgcagtc tcagtgccca cctgtttgtc agtaactctg 1320
aaggggctga cattttactg acttgcaaac aaataagcta actttccaga gttttgtgaa 1380
tgctggcaga gtccatgaga ctctgagtc agaggcaaag gcttttactg ctcacagctt 1440
agcagacagc atgaggttca tgttcacatt agtacacctt gccccccca aatctttag 1500
ggtgaccaga gcagtctagg tggatgctgt gcagaagggg tttgtgccac tggtgagaaa 1560
cctgagatta ggaatcctca atcttatact gggacaactt gcaaacctgc tcagccttg 1620
tctctgatga agatattatc ttcattgatc tggattgaaa acagacctac tctggaggaa 1680

FIGURE 4B

catattgtat cgattgtcct tgacagtaaa caaatctgtt gtaagagaca ttatctttat 1740
tatctaggac agtaagcaag cctggatctg agagagatat catcttgcaa ggatgcctgc 1800
tttaciaaaca tccttgaaac aacaatccag aaaaaaaaaag gtgttgctgt ctttgctcag 1860
aagacacaca gatacgtgac agaaccatgg agaattgcct cccaacgctg ttcagccaga 1920
gccttcacc cttgtctgca ggacagtctc aacgttccac cattaataac ttcttctatc 1980
acatcctgct tctttatgcc taaccaaggt tctaggtccc gatcgactgt gtctggcagc 2040
actccactgc caaaccaga ataaggcagc gctcaggatc ccgaaggggc atggctgggg 2100
atcagaactt ctgggttga gtgaggagt ggtccaccct cttgaatttc aaaggaggaa 2160
gaggctggat gtgaaggtag tgggggaggg aaagtgtcag ttccgaactc ttaggtcaat 2220
gagggaggag actggttaagg tcccagctcc cgaggtagct atgtgggaat ggcctaagaa 2280
tctcatatcc tcaggaagaa ggtgctggaa tcctgagggg tagagtctct ggtatatttg 2340
tggttaagg ctctttggcc cctgaaggca gaggctggaa ccattaggtc cagggtttgg 2400
ggtgatagta atgggatctc ttgattctc aagagtctga ggatcgaggg ttgccattc 2460
ttccatcttg ccacctaatc ctactccac ttgagggat caccagccct tctagctcca 2520
tgaaggctcc ctgggcaagc acaatctgag catgaaagat gcccagagg ccttgggtgt 2580
catccactca tcatccagca tcacactctg aggggtgtggc cagcaccatg acgtcatgtt 2640
gctgtgacta tccctgcagc gtgcctctcc agccacctgc caaccgtaga gctgccatc 2700
ctcctctggt gggagtggcc tgcattgtgc caggctgagg cctagtgtca gacagggagc 2760
ctggaatcat agggatccag gactcaaaag tgctagagaa tggccatag tcaccatcca 2820
tgaaatctca agggcttctg ggtggagggc acaggacact gaacttatgg tttccaagt 2880
ctattgctct cccaagttag tctcccagat acgaggcact gtgccagcat cagccttatc 2940
tccaccacat cttgtaaaag gactaccag ggccctgatg aacaccatgg tgtgtacagg 3000
agtagggggg ggaggcacgg actcctgtga ggtcacagcc aaggagcat catcatgggt 3060
ggggaggagg caatggacag gcttgagaac ggggatgtgg ttgtatttgg tttctttgg 3120
ttagataaag tgctgggtat aggatgaga gtggagtatg aagaccagtt aggatggagg 3180
atcagattgg agttgggtta gataaagtgc tgggtatagg attgagagt gagtatgaag 3240
accagttagg atggaggatc agattggagt tgggttagag atggggtaaa attgtgtcc 3300
ggatgagttt gggattgaca ctgtggaggt gggttgggat ggcatggctt tgggatggaa 3360

FIGURE 4C

atagatttgt tttgatgttg gctcagacat ccttggggat tgaactgggg atgaagctgg 3420
gtttgatttt ggaggtagaa gacgtggaag tagctgtcag atttgacagt ggccatgagt 3480
tttgtttgat ggggaatcaa acaatggggg aagacataag ggttggttg ttaggttaaag 3540
ttgcgttggg ttgatgggtt cggggctgtg tataatgcag ttggattggt ttgtattaaa 3600
ttgggttggg tcaggttttg gttgaggatg agttgaggat atgcttgggg acaccggatc 3660
catgaggttc tcaactggagt ggagacaaac ttcctttcca ggatgaatcc agggaagcct 3720
taattcacgt gtaggggagg tcaggccact ggctaagtat atccttccac tccagctcta 3780
agatggtctt aaattgtgat tatctatata cacttctgtc tccctcactg tgcttgaggt 3840
ttacctgac actcaactag aaacagggga agattttatc aaattctttt tttttttttt 3900
ttttttttga gacagagtct cactctgttg cccaggctgg agtgcagtgg cgcagtctcg 3960
gctcactgca acctctgcct cccaggttca agtgattctc ctgcctcagc ctctgagtt 4020
gctgggatta caggcatgca gcaccatgcc cagctaattt ttgtattttt agtagagatg 4080
gggtttcacc aatgtttgcc aggtggcct cgaactcctg acctggtgat ccacctgcct 4140
cagcctcca aagtgttggg attacaggcg tcagccaccg cgcccagcca cttttgtcaa 4200
attcttgaga cacagctcgg gctggatcaa gtgagctact ctggttttat tgaacagctg 4260
aaataacca ctttttgga attgatgaaa tcttacggag ttaacagtgg aggtaccagg 4320
gctcttaaga gttcccatt ctctctgag actacaaatt gtgattttgc atgccacctt 4380
aatctttttt tttttttttt taaatcgagg ttctagctc attctatttc ccaggctgga 4440
gttcaatagc gtgatcacag ctcaactgtg ccttgaactc ctggccttaa gagattctcc 4500
tgcttcggtc tcccaatagc taagactaca gtagtccacc accatatcca gataattttt 4560
aaattttttg gggggccggg cacagtggct cagcctgta atcccaacac catgggaggc 4620
tgagatgggt ggatcacgag gtcaggaggt tgagaccagc ctgaccaaca tggtgaaact 4680
ctgtctctac taaaaaaaaa aaaaatagaa aaattagccg ggcgtggtgg cacacggcac 4740
ctgtaatccc agctactgag gaggtgagg caggagaatc acttgaacct agaaggcaga 4800
ggttgcaatg agccgagatt gcgccactgc actccagcct gggtgacaga gtgagactct 4860
gtctcaaaaa aaaaaattt tttttttttt ttgttagaga tggatcttgc tttgtttctc 4920
tggttggcct tgaactcctg gtttcaagt atcctctac cttggcctcg gaaagtgttg 4980
ggattacagg cgtgagccac catgactgac ctgtcgttaa tcttgaggta cataaacctg 5040
gctcctaaag gctaaaggct aaatatttgt tggagaaggg gcattggatt ttgcatgagg 5100

FIGURE 4D

atgattctga cctgggaggg caggtcagca ggcattctctg ttgcacagat agagtgtaca 5160
ggctctggaga acaaggagtg gggggttatt ggaattccac attgtttgct gcacgttgga 5220
ttttgaaatg ctagggaact ttgggagact catatttctg ggctagagga tctgtggacc 5280
acaagatctt tttatgatga cagtagcaat gtatctgtgg agctggattc tgggttggga 5340
gtgcaaggaa aagaatgtac taaatgccaa gacatctatt tcaggagcat gaggaataaa 5400
agttctagtt tctggtctca gagtgtgca gggatcaggg agtctcaca tctctgagt 5460
gctggtgtct tagggcacac tgggtcttgg agtgcaaagg atctaggcac gtgaggcttt 5520
gtatgaagaa tcggggatcg taccacccc ctgtttctgt ttcattctgg gcatgtctcc 5580
tctgcctttg tcccctagat gaagtctcca tgagctacaa gggcctggtg catccagggt 5640
gatctagtaa ttgcagaaca gcaagtgcta gctctccctc cccttcaca gctctgggtg 5700
tgaggagggg ttgtccagcc tccagcagca tggggagggc cttggtcagc ctctgggtgc 5760
cagcagggca gggcgaggat cctggggaat gaaggtttta tagggctcct gggggaggct 5820
ccccagcccc aagctt 5836

FIGURE 5A

aagcttttta gtgctttaga cagtgaactg gtctgtctaa cccaagtac ctgggtcca	60
tactcagccc cagaagtga ggggaagct gggggagcc aaaccaggca agcctaccct	120
cagggctccc agtggcctga gaaccattgg acccaggacc cattacttct agggtaagga	180
aggtacaaac accagatcca accatggctt ggggggacag ctgtcaaagt cctaaaaata	240
tacctgggag aggagcaggc aaactatcac tgccccaggt tctctgaaca gaaacagagg	300
ggcaacccaa agtccaaatc caggtgagca ggtgcaccaa atgccagag atatgacgag	360
gcaagaagtg aaggaaccac ccctgcatca aatgttttgc atgggaagga gaagggggtt	420
gctcatgttc ccaatccagg agaatgcatt tgggatctgc cttcttctca ctccttggtt	480
agcaagacta agcaaccagg actctggatt tggggaaaga cgtttatttg tggaggccag	540
tgatgacaat cccacgaggc cctaggtgaa gagggcagga aggctcgaga cactggggac	600
tgagtgaaaa ccacacccat gatctgcacc acccatggat gctccttcat tgctcacctt	660
tctgttgata tcagatggcc ccattttctg taccttcaca gaaggacaca ggctagggtc	720
tgtgcatggc cttcatcccc ggggcatgt gaggcagca ggtgggaaag atcatgggtc	780
ctcctgggtc ctgcagggcc agaacattca tcaccatac tgacctcta gatgggaatg	840
gcttccctgg ggctgggcca acggggcctg ggcaggggag aaaggacgtc aggggacagg	900
gaggaagggt catcgagacc cagcctggaa ggttctgtc tctgaccatc caggatttac	960
ttccctgcat ctaccttggt tcattttccc tcagcaatga ccagctctgc ttctgatct	1020
cagcctccca ccctggacac agcaccacag tccctggccc ggctgcatcc acccaatacc	1080
ctgataaccc aggaccatt acttctaggg taaggagggt ccaggagaca gaagctgagg	1140
aaaggctctga agaagtcaca tctgtcctgg ccagagggga aaaaccatca gatgctgaac	1200
caggagaatg ttgaccagg aaagggaccg aggacccaag aaaggagtca gaccaccagg	1260
gtttgcctga gaggaaggat caaggccccg agggaaagca gggctggctg catgtgcagg	1320
acactgggtg ggcatatgtg tcttagattc tccctgaatt cagtgtccct gccatggcca	1380
gactctctac tcaggcctgg acatgctgaa ataggacaat ggccttgctc tctctcccca	1440
ccatttgga agagacataa aggacattcc aggacatgcc ttcctgggag gtccagggtc	1500
tctgtctcac acctcaggga ctgtagttac tgcatcagcc atggtaggtg ctgatctcac	1560
ccagcctgtc caggcccttc cactctccac tttgtgacca tgtccaggac caccctcag	1620
atcctgagcc tgcaaatacc cccttgctgg gtgggtggat tcagtaaaca gtgagctcct	1680

FIGURE 5B

atccagcccc cagagccacc tctgtcacct tctgtctggg catcatccca ccttcacaag	1740
cactaaagag catggggaga cctggctagc tgggtttctg catcaciaag aaaataatcc	1800
cccaggttcg gattcccagg gctctgtatg tggagctgac agacctgagg ccaggagata	1860
gcagagggtca gccctaggga ggggtgggtca tccaccaggg ggacaggggt gcaccagcct	1920
tgctactgaa agggcctccc caggacagcg ccatcagccc tgcttgagag ctttgctaaa	1980
cagcagtcag aggaggccat ggagtggtg gagctcctgc tccaggcccc aacagaccag	2040
accaacagca caatgcagtc cttccccaac gtcacagggt accaaaggga aactgagggtg	2100
ctacctaacc ttagagccat caggggagat aacagcccaa tttcccaaac aggccagttt	2160
caatcccatg acaatgacct ctctgtctc attcttccca aaataggacg ctgattctcc	2220
cccaccatgg atttctccct tgtcccggga gccttttctg cccctatga tctgggcact	2280
cctgacacac acctcctctc tggtagcata tcagggtccc tcactgtcaa gcagtccaga	2340
aaggacagaa ccttgagacag cgcccatctc agcttcaccc ttctccttc acagggttca	2400
gggcaaagaa taaatggcag aggccagtga gccacagagt ggtgacaggc agtgaccag	2460
gggcagatgc ctggagcagg agctggcggg gccacaggga gaaggtgatg caggaaggga	2520
aaccagaaa tgggcaggaa aggaggacac aggtctctgt gggctgcagc ccagggttgg	2580
actatgagtg tgaagccatc tcagcaagta aggccagggt ccatgaacaa gagtgggagc	2640
acgtggcttc ctgctctgta tatgggtgg gggattccat gcccataga accagatggc	2700
cggggttcag atggagaagg agcaggacag gggatcccca ggataggagg accccagtgt	2760
ccccaccag gcagggtgact gatgaatggg catgcagggt cctcctgggc tgggctctcc	2820
ctttgtccct caggattcct tgaaggaaca tccggaagcc gaccacatct acctggtggg	2880
ttctggggag tccatgtaaa gccaggagct tgtgttgcta ggaggggtca tggcatgtgc	2940
tgggggcacc aaagagagaa acctgagggc aggcaggacc tggctctgag aggcattggga	3000
gccagatgg ggagatggat gtcaggaaag gctgccccat caggagggt gatagcaatg	3060
gggggtctgt gggagtgggc acgtgggatt ccctgggctc tgccaagttc cctcccatag	3120
tcacaacctg gggacactgc ccatgaaggg gcgcctttgc ccagccagat gctgctggtt	3180
ctgcccattc actaccctct ctgctccagc cactctgggt ctttctccag atgccctgga	3240
cagccctggc ctgggctgt cccctgagag gtgttgggag aagctgagtc tctggggaca	3300
ctctcatcag agtctgaaag gcacatcagg aaacatccct ggtctccagg actaggcaat	3360

FIGURE 5C

gaggaaaggg cccagctcc tccctttgcc actgagaggg tcgaccctgg gtggccacag	3420
tgacttctgc gtctgtccca gtcaccctga aaccacaaca aaacccagc cccagaccct	3480
gcaggtacaa tacatgtggg gacagtctgt acccagggga agccagttct ctcttcctag	3540
gagaccgggc ctcagggtg tgccggggc aggcggggc agcacgtgcc tgtccttgag	3600
aactcgggac ctttaagggtc tctgctctgt gaggcacagc aaggatcctt ctgtccagag	3660
atgaaagcag ctctgcccc tcctctgacc tcttcctcct tcccaaactt caaccaacaa	3720
ataggtgttt caaatctcat catcaaactt tcatccatcc acatgagaaa gcttaaaacc	3780
caatggattg acaacatcaa gagttggaac aagtggacat ggagatgtta cttgtgaaa	3840
tttagatgtg ttcagctatc gggcaggaga atctgtgtca aattccagca tggttcagaa	3900
gaatcaaaaa gtgtcacagt ccaaagtgc aacagtgcag gggataaaac tgtggtgcat	3960
tcaaaactgag ggatattttg gaacatgaga aaggaagga ttgctgtgc acagaacatg	4020
gatgatctca cacatagagt tgaaagaaag gagtcaatcg cagaatagaa aatgatcact	4080
aattccacct ctataaagtt tccaagagga aaaccaatt ctgctgctag agatcagaat	4140
ggaggtgacc tgtgccttgc aatggctgtg aggtcacgg gagtgtcact tagtgacggc	4200
aatgtgccgt atcttaactt gggcagggtt ttcatgagca cataggaatg cagacattac	4260
tgtgtgttc attttacttc accggaaaag aagaataaaa tcagccgggc gcggtggctc	4320
acgcctgtaa tcccagcact ttagaaggct gaggtgggca gattacttga ggtcaggagt	4380
tcaagaccac cctggccaat atggtgaaac cccggctcta ctaaaaatac aaaaattagc	4440
tgggcatggt ggtgcgcgcc tgtaatecca gctactcggg aggtgaggc tggacaattg	4500
cttggacca ggaagcagag gttgcagtga gccaagattg tgccactgca ctccagcttg	4560
ggcaacagag ccagactctg taaaaaaaaa aaaaaaaaaa aaaaaagaa agaaagaaaa	4620
agaaaagaaa gtataaaatc tctttgggtt aacaaaaaaaa gatccacaaa acaaacacca	4680
gctcttatca aacttacaca actctgccag agaacaggaa acacaaatac tcattaactc	4740
acttttgtgg caataaaacc ttcatgtcaa aaggagacca ggacacaatg aggaagtaaa	4800
actgcaggcc ctacttgggt gcagagaggg aaaatccaca aataaaacat taccagaagg	4860
agctaagatt tactgcattg agttcattcc ccaggtatgc aaggtgattt taacacctga	4920
aaatcaatca ttgcctttac tacatagaca gattagctag aaaaaatta caactagcag	4980
aacagaagca atttggcctt cctaaaattc cacatcatat catcatgatg gagacagtgc	5040
agacgccaat gacaataaaa agagggacct ccgtcacccg gtaaacatgt ccacacagct	5100

FIGURE 5D

ccagcaagca cccgtcttcc cagtgaatca ctgtaacctc ccctttaatc agccccaggg	5160
aaggctgcct gcgatggcca cacaggctcc aaccctggg cctcaacctc ccgcagaggg	5220
tctcctttgg ccaccccatg gggagagcat gaggacaggg cagagccctc tgatgccac	5280
acatggcagg agctgacgcc agagccatgg gggctggaga gcagagctgc tggggtcaga	5340
gcttcctgag gacacccagg cctaaggga ggcagctccc tggatggggg caaccaggct	5400
ccgggctcca acctcagagc ccgcatggga ggagccagca ctctaggcct ttcctagggt	5460
gactctgagg ggaccctgac acgacaggat cgctgaatgc acccgagatg aagggggccac	5520
cacgggaccc tgctctcgtg gcagatcagg agagagtggg acaccatgcc agggcccat	5580
ggcatggctg cgactgaccc agggcactcc cctgcatgca tcagcctcgg taagtcacat	5640
gaccaagccc aggaccaatg tggaaggaag gaaacagcat cccctttagt gatggaaccc	5700
aaggctcagtg caaagagagg ccatgagcag ttaggaaggg tggccaacc tacagcacia	5760
accatcatct atcataagta gaagccctgc tccatgaccc ctgcatttaa ataaacgttt	5820
gttaaatgag tcaaattccc tcacatgag agctcacctg tgtgtaggcc catcacacac	5880
acaaacacac acacacacac acacacacac acacacacac acagggaaag tgcaggatcc	5940
tggacagcac caggcaggct tcacaggcag agcaaacagc gtgaatgacc catgcagtgc	6000
cctgggcccc atcagctcag agaccctgtg agggctgaga tggggctagg caggggagag	6060
acttagagag ggtggggcct ccagggaggg ggctgcaggg agctgggtac tgccctccag	6120
ggagggggct gcagggagct ggggtactgcc ctccagggag ggggctgcag ggagctgggt	6180
actgccctcc agggaggggg ctgcagggag ctgggtactg ccctccaggg agggggctgc	6240
agggagctgg gtactgccct ccagggagggc agggagcactg ttcccaacag agagcacatc	6300
ttcctgcagc agctgcacag acacaggagc ccccatgact gccctgggcc aggggtgtgga	6360
ttccaaattt cgtgccccat tgggtgggac ggagggtgac cgtgacatcc aaggggcatc	6420
tgtgattcca aacttaaact actgtgccta caaatagga aataacccta ctttttctac	6480
tatctcaaat tccctaagca caagctagca ccctttaaat caggaagttc agtcactcct	6540
ggggtcctcc catgccccca gtctgacttg cagggtgcaca ggggtggctga catctgtcct	6600
tgtcctcct cttgggtcaa ctgccgcccc tcctgggggt gactgatggg caggacaagg	6660
gatactagag ctggccccat gattgacagg aaggcaggac ttggcctcca ttctgaagac	6720
taggggtgtc aagagagctg ggcattccac agagctgcac aagatgacgc ggacagaggg	6780

FIGURE 5E

tgacacaggg ctcagggctt cagacgggtc gggaggctca gctgagagtt cagggacaga	6840
cctgaggagc ctcagtggga aaagaagcac tgaagtggga agttctggaa tgttctggac	6900
aagcctgagt gctctaagga aatgctccca ccccgatgta gcctgcagca ctggacggtc	6960
tgtgtacctc cccgctgccc atcctctcac agccccgcc tctagggaca caactcctgc	7020
cctaacatgc atctttcctg tctcattcca cacaaaaggy cctctggggt ccctgttctg	7080
cattgcaagg agtggaggtc acgttcccac agaccacca gcaacagggt cctatggagg	7140
tgcggtcagg aggatcacac gtcccccat gcccaggga ctgactctgg gggatgagga	7200
ttggcctgga ggccactggt cccctctgtc cctgaggga atctgcaccc tggaggctgc	7260
cacatccctc ctgattcttt cagctgaggg cccttcttga aatcccaggg aggactcaac	7320
ccccactggg aaaggcccag tgtggacggg tccacagcag ccagctaag gcccttggac	7380
acagatcctg agtgagagaa cctttaggga cacagggtga cgccatgtc ccagtgccc	7440
acacagagca ggggcatctg gaccctgagt gtgtagctcc cgcgactgaa ccagccctt	7500
ccccaatgac gtgaccctg ggggtggctcc aggtctccag tccatgccac caaaatctcc	7560
agattgaggg tcctcccttg agtccctgat gcctgtccag gagctgccc ctgagcaaat	7620
ctagagtga gagggctggg attgtggcag taaaagcagc cacatttgtc tcaggaagga	7680
aagggaggac atgagctcca ggaagggcga tggcgtcctc tagtgggggc ctctgttaa	7740
tgagcaaaaa ggggccagga gagttgagag atcagggtc gccttggact aaggctcaga	7800
tggagaggac tgaggtgcaa agagggggct gaagtagggg agtggtcggg agagatggga	7860
ggagcaggtg aggggaagcc ccaggagggc cgggggaggg tacagcagag ctctccactc	7920
ctcagcattg acatttgggg tggctgtgct agtgggggtc tgtaagtgtt aggggtgtca	7980
gcaccatctg gggactctac ccactaaatg ccagcaggac tcctcccca agctctaaca	8040
accaacaatg tctccagact ttccaaatgt cccctggaga gcaaaattgc ttctggcaga	8100
atcactgatc tacgtcagtc tctaaaagtg actcatcagc gaaatccttc acctcttggg	8160
agaagaatca caagtgtgag aggggtagaa actgcagact tcaaaatctt tccaaaagag	8220
ttttacttaa tcagcagttt gatgtcccag gagaagatac atttagagtg ttttagagtg	8280
atgccacatg gctgcctgta cctcacagca ggagcagagt gggttttcca agggcctgta	8340
accacaactg gaatgacact cactgggtta cattacaaag tggaatgtgg ggaattctgt	8400
agactttggg aagggaaatg tatgacgtga gccacagcc taaggcagtg gacagtccac	8460
tttgaggctc tcaccatcta ggagacatct cagccatgaa catagccaca tctgtcatta	8520

FIGURE 5F

gaaaacatgt tttattaaga ggaaaaatct aggctagaag tgctttatgc tcttttttct	8580
ctttatgttc aaattcatat acttttagat cattccttaa agaagaatct atccccctaa	8640
gtaaatgtta tcaactgactg gatagtgttg gtgtctcact cccaaccctt gtgtggtgac	8700
agtgccctgc tccccagcc ctgggccctc tctgattcct gagagctttg ggtgctcctt	8760
cattaggagg aagagaggaa ggtgtttttt aatattctca ccattcaccc atccacctct	8820
tagacactgg gaagaatcag ttgccactc ttggatttga tctcgaatt aatgacctct	8880
atttctgtcc cttgtccatt tcaacaatgt gacaggccta agagggtgcct tctccatgtg	8940
atttttgagg agaaggttct caagataagt tttctcacac ctctttgaat tacctccacc	9000
tgtgtcccca tcaccattac cagcagcatt tggacccttt ttctgttagt cagatgcttt	9060
ccacctcttg aggggtgtata ctgtatgctc tctacacagg aatatgcaga ggaaatagaa	9120
aaagggaaat cgcattacta ttcagagaga agaagacctt tatgtgaatg aatgagagtc	9180
taaaatccta agagagccca tataaaatta ttaccagtgc taaaactaca aaagttacac	9240
taacagtaaa ctagaataat aaaacatgca tcacagtgc tggtaaagct aaatcagata	9300
tttttttctt agaaaaagca ttccatgtgt gttgcagtga tgacaggagt gcccttcagt	9360
caatatgctg cctgtaattt ttgttccttg gcagaatgta ttgtcttttc tccctttaaa	9420
tcttaaatgc aaaactaaag gcagctcctg ggccccctcc ccaaagtcag ctgcctgcaa	9480
ccagccccac gaagagcaga ggcctgagct tccctggtca aaataggggg ctagggagct	9540
taaccttgct cgataaagct gtgttcccag aatgtcgtc ctgttcccag gggcaccagc	9600
ctggagggtg gtgagcctca ctggtggcct gatgcttacc ttgtgccctc acaccagtgg	9660
tcaactggaac cttgaacact tggctgtcgc ccggatctgc agatgtcaag aacttctgga	9720
agtcaaatta ctgcccactt ctccagggca gatacctgtg aacatccaaa accatgccac	9780
agaacctgc ctggggtcta caacacatat ggactgtgag caccaagtcc agccctgaat	9840
ctgtgaccac ctgccaagat gccctaact gggatccacc aatcactgca catggcaggc	9900
agcgaggctt ggagggtgctt cgccacaagg cagccccaat ttgctgggag tttcttgga	9960
cctggtagtg gtgaggagcc ttgggacct caggattact ccccttaagc atagtgggga	10020
cccttctgca tccccagcag gtgccccgct cttcagagcc tctctctctg aggtttaccc	10080
agacccctgc accaatgaga ccatgctgaa gcctcagaga gagagatgga gctttgacca	10140
ggagccgctc ttccttgagg gccagggcag ggaaagcagg aggcagcacc aggagtggga	10200

FIGURE 5G

acaccagtgt ctaagccct gatgagaaca ggggtgtctc tcccatatgc ccataccagg 10260
 cctgtgaaca gaatcctcct tctgcagtga caatgtctga gaggacgaca tgtttcccag 10320
 cctaacgtgc agccatgcc atctaccac tgcctactgc aggacagcac caaccaggga 10380
 gctgggaagc tgggagaaga catggaatac ccatggcttc tcaccttccct ccagtccagt 10440
 gggcaccatt tatgcctagg acaccacct gccggcccca ggctcttaag agttagggtca 10500
 cctaggtgcc tctgggaggc cgaggcagga gaattgcttg aaccggggag gcagagggtg 10560
 cagtgagccg agatcacacc actgcactcc agcctgggtg acagaatgag actctgtctc 10620
 aaaaaaaaag agaaagatag catcagtggc taccaagggc taggggcagg ggaagggtga 10680
 gagttaatga ttaatagtat gaagtttcta tgtgagatga tgaaaatgtt ctggaaaaa 10740
 aaatatagtg gtgaggatgt agaatttgt gaataaatt aacggcattt aattgtacac 10800
 ttaacatgat taatgtggca tttttatct tatgtatttg actacatcca agaaacctg 10860
 ggagagggaa agcccaccat gtaaaataca cccaccctaa tcagatagtc ctattgtac 10920
 ccaggtacag gccctcatg acctgcacag gaataactaa ggatttaagg acatgaggct 10980
 tcccagcaa ctgcaggtgc acaacataaa tgtatctgca aacagactga gagtaaagct 11040
 gggggcaca acctcagcac tgccaggaca cacacccttc tcgtggattc tgactttatc 11100
 tgaccggcc cactgtccag atctgttgt gggattggga caaggagggt cataaagcct 11160
 gtccccaggg cactctgtgt gagcacacga gacctccca cccccacc gttagggtctc 11220
 cacacataga tctgaccatt aggcattgtg aggaggactc tagcggggc tcagggatca 11280
 caccagagaa tcaggtacag agaggaagac ggggctcgag gagctgatgg atgacacaga 11340
 gcagggttcc tgcagtccac aggtccagct caccctgggt taggtgcccc atccccctga 11400
 tccaggcatc cctgacacag ctccctcccg gagcctctc ccaggtgaca catcagggtc 11460
 cctcactcaa gctgtccaga gagggcagca ccttgacag cgccacccc acttcactct 11520
 tcctccctca cagggtcag ggctcagggc tcaagtctca gaacaaatgg cagaggccag 11580
 tgagcccaga gatggtgaca gggcaatgat ccaggggcag ctgcctgaaa cgggagcagg 11640
 tgaagccaca gatgggagaa gatggttcag gaagaaaaat ccaggaatgg gcaggagagg 11700
 agaggaggac acaggctctg tggggctgca gccaggatg ggactaagtg tgaagacatc 11760
 tcagcagggtg aggccaggtc ccatgaacag agaagcagct cccacctccc ctgatgcacg 11820
 gacacacaga gtgtgtggtg ctgtgcccc agagtcgggc tctctgttc tgggtcccag 11880
 ggagtgagaa gtgagggtga cttgtccctg ctctctctg ctacccaac attcaccttc 11940

FIGURE 5H

tcctcatgcc cctctctctc aaatatgatt tggatctatg tccccgcca aatctcatgt	12000
caaattgtaa accccaatgt tggaggtggg gccttgtgag aagtgattgg ataatgcggg	12060
tggattttct gctttgatgc tgtttctgtg atagagatct cacatgatct ggttgtttaa	12120
aagtgtgtag cacctctccc ctctctctct ctctctctta ctcatgctct gccatgtaag	12180
acgttcctgt ttccccctca ccgtccagaa tgattgtaag ttttctgagg cctccccagg	12240
agcagaagcc actatgcttc ctgtacaact gcagaatgat gagcgaatta aacctctttt	12300
ctttataaat taccagctct caggtatttc tttatagcaa tgcgaggaca gactaataca	12360
atcttctact cccagatccc cgcacacgt tagccccaga catcactgcc cctgggagca	12420
tgcacagcgc agcctcctgc cgacaaaagc aaagtcacaa aagggtgacaa aaatctgcat	12480
ttggggacat ctgattgtga aagagggagg acagtacact tgtagccaca gagactgggg	12540
ctcaccgagc tgaaacctgg tagcactttg gcataacatg tgcagtaccc gtgttcaatg	12600
tctagagatc agtggtgagt aaaacagcct ggtctggggc cgctgctgtc cccacttccc	12660
tcctgtccac cagagggcgg cagagttcct cccaccctgg agcctcccca ggggctgctg	12720
acctccctca gccgggcca cagcccagca ggggtccacc tcacccgggt cacctcggcc	12780
cacgtcctcc tcgccctccg agtcctcac acggactctg tcagctctc cctgcagcct	12840
atcgccgcc cacctgaggc ttgtcggccg cccacttgag gcctgtcggc .tgccctctgc	12900
aggcagctcc tgtccctac acccctcct tccccgggt cagctgaaag ggcgtctccc	12960
agggcagctc cctgtgatct ccaggacagc tcagtctctc acaggctccg acgcccccta	13020
tgctgtcacc tcacagccct gtcattacca ttaactctc agtcccatga agttcactga	13080
gcgcctgtct cccggttaca ggaaaaactct gtgacaggga ccacgtctgt cctgctctct	13140
gtggaatccc agggcccagc ccagtgcctg acacggaaca gatgctccat aaatactggt	13200
taaatgtgtg ggagatctct aaaaagaagc atatcacctc cgtgtggccc ccagcagtca	13260
gagtctgttc catgtggaca caggggcaact ggcaccagca tgggaggagg ccagcaagtg	13320
cccgcggctg cccaggaat gaggcctcaa ccccagagc ttcagaaggg aggacagagg	13380
cctgcaggga atagatctc cggcctgacc ctgcagccta atccagagtt cagggtcagc	13440
tcacaccacg tcgaccctgg tcagcatccc tagggcagtt ccagacaagg ccggaggctct	13500
cctcttgccc tccagggggt gacattgcac acagacatca ctcaggaaac ggattcccct	13560
ggacaggaac ctggctttgc taagggaagtg gaggtggagc ctggtttcca tcccttgctc	13620

FIGURE 5I

caacagaccc ttctgatctc tcccacatac ctgctctgtt cttttctggg tcctatgagg	13680
accctgttct gccaggggtc cctgtgcaac tccagactcc ctctgtgtac caccatgggg	13740
aaggtggggt gatcacagga cagtcagcct cgcagagaca gagaccaccc aggactgtca	13800
gggagaacat ggacaggccc tgagccgcag ctccagccaac agacacggag agggagggtc	13860
cccctggagc cttccccaag gacagcagag cccagagtca cccacctccc tccaccacag	13920
tcctctcttt ccaggacaca caagacacct cccctccac atgcaggatc tggggactcc	13980
tgagacctct gggcctgggt ctccatccct gggtcagtgg cggggttggt ggtactggag	14040
acagagggct ggtccctccc cagccaccac ccagtgcagc tttttctagc cccagagcc	14100
acctctgtca ccttctgtt gggcatcatc ccaccttccc agagccctgg agagcatggg	14160
gagaccggg accctgtgtg gtttctctgt cacaaggaa aataatcccc ctggtgtgac	14220
agaccaagg acagaacaca gcagaggtca gcactgggga agacaggttg tcctcccagg	14280
ggatgggggt ccatccacct tgccgaaaag atttgtctga ggaactgaa atagaaggga	14340
aaaaagagga gggacaaaag aggcagaaat gagaggggag gggacagagg acacctgaat	14400
aaagaccaca cccatgacct acgtgatgct gagaagtact cctgccctag gaagagactc	14460
agggcagagg gaggaaggac agcagaccag acagtcacag cagccttgac aaaacgttcc	14520
tggaaactcaa gctcttctcc acagaggagg acagagcaga cagcagagac catggagtct	14580
ccctcgcccc ctccccacag atggtgcatc ccctggcaga ggctcctgct cacaggtgaa	14640
gggaggacaa cctgggagag ggtgggagga gggagctggg gtctcctggg taggacaggg	14700
ctgtgagacg gacagagggc tcctgttggg gcctgaatag ggaagaggac atcagagagg	14760
gacaggagtc acaccagaaa aatcaaattg aactggaatt ggaaaggggc aggaaaacct	14820
caagagttct attttctag ttaattgtca ctggccacta cgtttttaaa aatcataata	14880
actgcatcag atgacacttt aaataaaaac ataaccaggg catgaaacac tgtcctcatc	14940
cgcctaccgc ggacattgga aaataagccc caggctgtgg agggccctgg gaacctcat	15000
gaactcatcc acaggaatct gcagcctgtc ccaggcactg gggtgcaacc aagatc	15056

FIGURE 6A

gaattcagaa ataggggaag gttgaggaag gacactgaac tcaaagggga tacagtgatt 60
ggtttatttg tcttctcttc acaacattgg tgctggagga attcccaccc tgaggttatg 120
aagatgtctg aacacccaac acatagcact ggagatatga gctcgacaag agtttctcag 180
ccacagagat tcacagccta gggcaggagg acactgtacg ccaggcagaa tgacatggga 240
attgcgctca cgattggctt gaagaagcaa ggactgtggg aggtgggctt tgtagtaaca 300
agagggcagg gtgaactctg attcccatgg gggaatgtga tggtcctgtt acaaattttt 360
caagctggca ggggaataaaa cccattacgg tgaggacctg tggagggcgg ctgccccaac 420
tgataaagga aatagccagg tgggggcctt tccattgta ggggggacat atctggcaat 480
agaagccttt gagacccttt aggggtacaag tactgaggca gcaaataaaa tgaaatctta 540
tttttcaact ttatactgca tgggtgtgaa gatataattg tttctgtaca gggggtgagg 600
gaaaggaggg gaggaggaaa gttcctgcag gtctggtttg gtcttgtgat ccaggggggc 660
ttggaactat ttaaattaaa ttaaattaaa acaagcgact gttttaaatt aaattaaatt 720
aaattaaatt ttactttatt ttactttaag ttctgggcta catgtgcagg acgtgcagct 780
ttgttacata ggtaaacgtg tgccatgggt gtttgctgta cctatcaacc catcacctag 840
gtattaagcc cagcatgcat tagctgtttt tcctgacgct ctccctctcc ctgactccca 900
caacaggccc cagtgtgtgt tgttcccctc cctgtgtcca tgtgttctca ttgttcagct 960
cccacttata agtgagaaca tgtggtgttt gggtttctgt ttctgtgtta gtttgctgag 1020
gataatggct tccacctcca tccatgttcc tgcaaaggac gtgatcttat tcttttttat 1080
ggttgcatag aaattgtttt tacaatcca attgatattg tatttaatta caagttaatc 1140
taattagcat actagaagag attacagaag atattaggta cattgaatga ggaaatatat 1200
aaaataggac gaaggtgaaa tattaggtag gaaaagtata atagttgaaa gaagtaaaaa 1260
aaaatatgca tgagtagcag aatgtaaaag aggtgaagaa cgtaatatgtg actttttaga 1320
ccagattgaa ggacagagac agaaaaattt taaggaattg ctaaaccatg tgagtgttag 1380
aagtacagtc aataacatta aagcctcagg aggagaaaag aataggaaaag gaggaaatat 1440
gtgaataaat agtagagaca tgtttgatgg attttaaaat atttgaaaga cctcacatca 1500
aaggattcat accgtgccat tgaagaggaa gatggaaaag ccaagaagcc agatgaaagt 1560
tagaaatatt attggcaaag cttaaatgtt aaaagtccta gagagaaaag atggcagaaa 1620
tattggcggg aaagaatgca gaacctagaa tataaattca tccaacagct ttggtagtgt 1680

FIGURE 6B

gcagctgtag ccttttctag ataatacact attgtcatcac atcgcttaag cgagtgtaaa 1740
atggtctcct cactttatct atttatatat ttatttagtt ttgagatgga gcctcgctct 1800
gtctcctagg ctggagtcca atagtgcgat accactcact gcaacctctg cctcctctgt 1860
tcaagtgatt ttcttacctc agcctcccga gtagctggga ttacaggtgc gtgccaccac 1920
acccggctaa tttttgtatt tttttagag acggggtttt gccatgttgg ccaggctggt 1980
cttgaactcc tgacatcagg tgatccacct gccttggcct cctaaagtgc tgggattaca 2040
ggcatgagcc accgtgcccc accactttat ttatttttta tttttatttt taaatttcag 2100
cttctatttg aaatacaggg ggcacatata taggattggt acatgggtat attgaactca 2160
ggtagtgatc atactaccca acaggtaggt tttcaaccca ctccccctct ttctctcccc 2220
attctagtag tgtgcagtgt ctattgttct catgtttatg tctatgtgtg ctccagggtt 2280
agctcccacc tgtaagtgag aacgtgtggt atttgatttt ctgtccctgt gtttaattcac 2340
ttaggattat ggcttcacgc tccattcata ttgctgtaaa ggatatgatt catttttcat 2400
ggccatgcag tattccatat tgcgtataga tcacattttc tttctttttt ttttttgaga 2460
cggagtcttg ctttgctgcc taggctggag tgcagtagca cgatctcggc tcaactgcaag 2520
cttcacctcc ggggttcacg tcattcttct gtctcagctt cccaagtagc tgggactaca 2580
ggcgcccgcc accacgtccg gctaattttt ttgtgtgttt ttagtagaga tgggggtttc 2640
actgtgttag ccaggatggt cttgatctcc tgacctgtg gtccacctgc ctcggtctcc 2700
caaagtgtg ggattacagg ggtgagccac tgcgcccggc ccatatatac cacattttct 2760
ttaaccaatc caccattgat gggcaactag gtagattcca tggattccac agttttgcta 2820
ttgtgtgcag tgtggcagta gacatatgaa tgaatgtgtc tttttggtat aatgatttgc 2880
attccttttg gtatacagtc attaatagga gtgctgggtt gaacggtggc tctgtttaaa 2940
attcctttgag aattttccaa actgtttgcc atagagagca aactaattta catttccacg 3000
aacagtatat aagcattccc ttttctccac agctttgtca tcatgggttt ttttttctt 3060
tattttaaaa aagaatatgt tgttgttttc ccagggtaca tgtgcaggat gtgcaggttt 3120
gttacatagg tagtaaacgt gagccatggt ggtttgctgc acctgtcaac ccattacctg 3180
ggatatgaag cctgcctgca ttagctcttt tcctaatgc tctcactact gccccacct 3240
caccctgaca gggcaaacag acaacctaca gaatgggagg aaatttttgc aatctattca 3300
tctgacaaag gtcaagaata tccagaatct acaagggaact taagcaaatt tttacttttt 3360

FIGURE 6C

aataatagcc actctgactg gcgtgaaatg gtatctcatt gtggttttca tttgaatttc 3420
tctgatgac agtgacgatg agcatttttt catatttggt ggctgcttgt acgtcttttg 3480
agaagtgtct cttcatgcct tttggccact ttaatgggat tttttttgct tttttagttt 3540
aagttcctta tagattctgg atattagact tcttattgga tgcatagttt gtgaatactc 3600
tcttccattc tgtagggtgt ctgtttactc tattgatggc ttcttttgct gtgccgaagc 3660
atcttagttt aattagaaac cacctgccaa tttttgtttt tgttgcaatt gcttttgggg 3720
acttagtcat aaactctttg ccaaggctcg ggtcaagaag agtatttcct aggttttctt 3780
ctagaatttt gaaagtctga atgtaaacad ttgcattttt aatgcatctt gagttagttt 3840
ttgtatatgt gaaaggctca ctctcatttt ctttccctct ttctttcttt ctttcttttc 3900
tttctttctt tctttctttc tttctttctt tctttctttc tttctttttg tcttctttc 3960
tttctttctt tcttctttct tctctctttc tttttttttt ttgatggagt attgctctgt 4020
tgcccaggct gcagtgcagc ggcacgatct cggctcactg caacctctgc ctccctgggtt 4080
caactgattc tcttgcata gccttccaag tagctgggat tataggcgcc cgccaccacg 4140
cccgaactaat ttttgtattt ttagtagaga cgggggttggt ccatgttggc caggctgggtt 4200
tgaaactcct gacctcaaac gatctgcctg ccttggcctc ccaaagtgtt gggattacag 4260
gtgtgagcca ctgtgccag ccaagaatgt cattttctaa gaggtccaag aacctcaaga 4320
tattttggga ccttgagaag agaggaattc atacaggtat tacaagcaca gcctaattggc 4380
aaatctttgg catggcttgg cttcaagact ttaggtcttt aaaagtcgaa tccaaaaatt 4440
tttataaaag ctccagctaa gctaccttaa aaggggcctg tatggctgat cactcttctt 4500
gctatacttt acacaaataa acaggccaaa tataatgagg caaaaattta ttttgcaaatt 4560
aaattggtcc tgctatgatt tactcttggg aagaacaggg aaaatagaga aaaatttaga 4620
ttgcatctga cttttttttc tgaattttta tatgtgccta caatttgagc taaatcctga 4680
attattttct ggttgcaaaa actctctaaa gaagaacttg gttttcattg tcttcgtgac 4740
acatttatct ggctctttac tagaacagct ttcttgtttt tgggtgttcta gcttgtgtgc 4800
cttacagttc tactcttcaa attattgtta tgtgtatctc atagttttcc ttcttttgag 4860
aaaactgaag ccattggtatt ctgaggacta gagatgactc aacagagctg gtgaatctcc 4920
tcatatgcaa tccactgggc tcgatctgct tcaaattgct gatgactgc tgctaaagct 4980
atacatttta aacctcact aaaggatcag ggaccatcat ggaagaggag gaaacatgaa 5040
attgtaagag ccagattcgg ggggtagagt gtggagggtca gagcaactcc accttgaata 5100

FIGURE 6D

agaaggtaaa gcaacctatc ctgaaagcta acctgccatg gtggcttctg attaacctct 5160
gttctaggaa gactgacagt ttgggtctgt gtcattgccc aaatctcatg ttaaattgta 5220
atccccagtg ttcggagggtg ggacttggtg gtaggtgatt cggtcatggg agtagatttt 5280
cttctttgtg gtgttacagt gatagtgagt gagttctcgt gagatctggt catttaaaag 5340
tgtgtggccc ctcccctccc tctcttggtc ctctactgc catgtaagat acctgctcct 5400
gctttgcctt ctaccataag taaaagcccc ctgaggcctc cccagaagca gatgccacca 5460
tgcttcctgt acagcctgca gaaccatcag ccaattaaac ctcttttctg tataaattac 5520
cagtcttgag tatctcttta cagcagtgtg agaacggact aatacaaggg tctccaaaat 5580
tccaagttha tgtattcttt cttgccaaat agcaggattt taccataaat cctgtcctta 5640
ggcacaacaa ccttgatggc atcgtacttc aattgtctta cacattcctt ctgaatgact 5700
cctcccctat ggcatataag ccctgggtct tgggggataa tggcagaggg gtccaccatc 5760
ttgtctggct gccacctgag acacggacat ggcttctggt ggtaagtctc tattaatgt 5820
ttctttctaa gaaactggat ttgtcagctt gtttctttgg cctctcagct tcctcagact 5880
ttggggtagg ttgcacaacc ctgccacca cgaaacaaat gttaaatatg ataaatatgg 5940
atagatataa tccacataaa taaaagctct tggagggccc tcaataattg ttaagagtgt 6000
aaatgtgtcc aaagatggaa aatgtttgag aactactgtc ccagagattt tcctgagttc 6060
tagagtgtgg gaatatagaa cctggagctt ggcttcttca gcctagaatc aggagtatgg 6120
ggctgaagtc tgaagcttgg cttcagcagt ttggggttgg cttccggagc acatatttga 6180
catgttgca ctgtgatttg gggtttggtt ttgtctctga atcctaattg ctgtccttga 6240
ggcatctaga atctgaaatc tgtggtcaga attctattat cttgagtagg acatctccag 6300
tcctggttct gccttctagg gctggagtct gtagtcagt acccggctct gcatttcaac 6360
ttcatataca gtgggtatc ttttggtcca tgtttcaacc aaacaaccga ataaaccatt 6420
agaaccttcc cccacttccc tagctgcaat gttaaaccct ggatttctgt ttaatagggt 6480
catatgaata atttcagcct gatccaactt tacattcctt ctaccgttat tctacacca 6540
ccttaaaaat gcattcccaa tatattccct ggattctacc tatatatggg aatcctggct 6600
ttgccagttt ctagtgcatt aacatacctg atttacatc tttacttta aagtggaaat 6660
aagagtcctt ctgcagagt caggagttct caagatggcc ctacttctg acatcaattg 6720
agatttcaag ggagtcgcca agatcatcct caggttcagt gattgctggt agccctcata 6780

FIGURE 6E

taactcaatg aaagctgtta tgctcatggc tatggtttat tacagcaaaa gaatagagat 6840
gaaaatctag caagggaaga gttgcatggg gcaaagacaa ggagagctcc aagtgcagag 6900
attcctgttg ttttctccca gtggtgtcat ggaaagcagt atcttctcca tacaatgatg 6960
tgtgataata ttcagtgtat tgccaatcag ggaactcaac tgagccttga ttatatggga 7020
gcttggttgc acagacatgt cgaccacctt catggctgaa ctttagtact tagccctcc 7080
agacgtctac agctgatagg ctgtaacca acattgtcac cataaatcac attgttagac 7140
tatccagtgt ggccaagct cccgtgtaa cacaggcact ctaaacaggc aggatatttc 7200
aaaagcttag agatgacctc ccaggagctg aatgcaaaga cctggcctct ttgggcaagg 7260
agaatccttt accgcacact ctccttcaca gggttattgt gaggatcaaa tgtggtcatg 7320
tgtgtgagac accagcacat gtctggtgtg ggagagtgc ttctatgtgt gctaacattg 7380
ctgagtgcga agaaagtatt aggcattggc ttcagcactc acagatgctc atctaactct 7440
cacaacatgg ctacagggtg ggcactacta gcctcatttg acagaggaaa ggactgtgga 7500
taagaagggg gtgaccaata ggtcagagtc attctggatg caaggggctc cagaggacca 7560
tgattagaca ttgtctgcag agaaattatg gctggatgct tctgccccgg aaagggggat 7620
gcactttcct tgaccccta tctcagatct tgactttgag gttatctcag acttctctca 7680
tgataccagg agcccatcat aatctctctg tgcctctccc ccttctcag tcttactgcc 7740
cactcttccc agctccatct ccagctggcc aggtgtagcc acagtaccta actctttgca 7800
gagaactata aatgtgtatc ctacagggga gaaaaaaaaa aagaactctg aaagagctga 7860
cattttaccg acttgcaaac acataagcta acctgccagt tttgtgctgg tagaactcat 7920
gagactcctg ggtcagaggc aaaagatttt attaccaca gctaaggagg cagcatgaac 7980
tttgtgttca catttggtca ctttgcccc caattcatat gggatgatca gagcagttca 8040
ggtggatgga cacaggggtt tgtggcaaag gtgagcaacc taggcttaga aatcctcaat 8100
cttataagaa ggtactagca aacttgcca gtctttgtat ctgacggaga tattatcttt 8160
ataattgggt tgaaagcaga cctactctgg aggaacatat tgtatttatt gtccctgaaca 8220
gtaaacaaat ctgctgtaaa atagacgtta actttattat ctaaggcagt aagcaaact 8280
agatctgaag gcgataccat cttgcaaggc tatctgctgt acaaatatgc ttgaaaagat 8340
ggtccagaaa agaaaacggt attattgcct ttgctcagaa gacacacaga aacataagag 8400
aaccatggaa aattgtctcc caacactgtt caccagagc cttccactct tgtctgcagg 8460
acagtcttaa catcccatca ttagtgtgtc taccacatct ggcttcaccg tgcctaacca 8520

FIGURE 6F

agatttctag gtccagttcc ccaccatgtt tggcagtgcc ccaactgccaa cccagaata 8580
agggagtgtc cagaattccg aggggacatg ggtggggatc agaacttctg ggcttgagtg 8640
cagagggggc ccatactcct tggttccgaa ggaggaagag gctggagggtg aatgtccttg 8700
gaggggagga atgtgggttc tgaactctta aatccccaag ggaggagact ggtaagggtcc 8760
cagcttccga ggtactgacg tgggaatggc ctgagagggtc taagaatccc gtatcctcgg 8820
gaaggagggg ctgaaattgt gaggggtga gttgcagggg tttgttagct tgagactcct 8880
tggtgggtcc ctgggaagca aggactggaa ccattggctc cagggtttg tgtgaaggta 8940
atgggatctc ctgattctca aagggtcaga ggactgagag ttgccatgc tttgatcttt 9000
ccatctactc cttactccac ttgagggtaa tcacctactc ttctagttcc acaagagtgc 9060
gcctgcgcga gtataatctg cacatgtgcc atgtcccag gcctggggca tcatccactc 9120
atcattcagc atctgcgcta tgcgggcgag gccggcgcca tgacgtcatg tagctgcgac 9180
tatccctgca gcgcgcctct cccgtcacgt cccaaccatg gagctgtgga cgtgcgtccc 9240
ctgggtgatg tggcctgcgt ggtgccaggc cggggcctgg tgtccgataa agatcctaga 9300
accacaggaa accaggactg aaagggtgta gagaatggcc atatgtcgt gtccatgaaa 9360
tctcaaggac ttctgggtgg agggcacagg agcctgaact tacgggttg cccaggtcca 9420
ctgtcctccc aagtgagtct ccagatacg aggcactgtg ccagcatcag cttcatctgt 9480
accacatctt gtaacaggga ctaccagga ccctgatgaa caccatgggtg tgtgcaggaa 9540
gaggggggtga aggcattggac tcctgtgtgg tcagagccca gagggggcca tgacgggtgg 9600
ggaggaggct gtggactggc tcgagaagtg ggatgtggtt gtgtttgatt tcctttggcc 9660
agataaagtg ctggatatag cattgaaaac ggagtatgaa gaccagttag aatggagggt 9720
caggttggag ttgagttaca gatggggtaa aattctgctt cggatgagtt tggggattgg 9780
caatctaaag gtggtttggg atggcatggc tttgggatgg aaataggttt gtttttatgt 9840
tggctgggaa ggggtgtggg attgaattgg ggatgaagta ggtttagttt tggagataga 9900
atacatggag ctggctattg catgcgagga tgtgcattag tttggtttga tctttaaata 9960
aaggaggcta ttagggttgt cttgaattag attaagttgt gttgggttga tgggttgggc 10020
ttgtgggtga tgtggttga ttgggctgtg ttaaattggt ttgggtcagg ttttggttga 10080
ggttatcatg gggatgagga tatgcttggg acatggattc aggtggttct cattcaagct 10140
gaggcaaatt tcctttcaga cggtcattcc agggaaacgag tggttgtgtg ggggaaatca 10200

FIGURE 6G

ggccactggc tgtgaatata cctctatcct ggtcttgaat tgtgattata tatgtccatt 10260
ctgtctcctt cactgtactt ggaattgata tggatcattca gctggaaatg ggggaagatt 10320
ttgtcaaatt cttgagacac agctgggtct ggatcagcgt aagccttctt tctggtttta 10380
ttgaacagat gaaatcacat tttttttttt aaaatcacag aaatcttata gagttaacag 10440
tggactctta taataagagt taacaccagg actcttattc ttgattcttt tctgagacac 10500
caaaatgaga tttctcaatg ccaccctaata tttttttttt tttttttttt tttttgagac 10560
acagtctggg tcttttgctc tgtcactcag gctggagcgc agtgggtgta tcatagctca 10620
ctgaaccctt gacctcctgg acttaaggga tcctcctgct tcagcctcct gagtagatgg 10680
ggctacaggt gcttgccacc acacctggct aattaaattt tttttttttt tttgtagaga 10740
aagggtctca ctttgttgcc ctggctgata ttgaacttct gacttcaagt gattcttcag 10800
ccttggaact ccaaagcact gggattgctg gcatgagcca ctcaccgtgc ctggcttgca 10860
gcttaattct ggagtgtata aacctggctc ctgatatgta gacatttcag tgagaaggag 10920
gcattggatt ttgcatgagg acaattctga cctaggaggg cagggtcaaca ggaatccccg 10980
ctgtacctgt acgttgata ggcatggaga atgaggagtg aggaggccgt accggaaccc 11040
catattgttt agtggacatt ggattttgaa ataataggga acttgggtctg ggagagtcatt 11100
atttctggat tggacaatat gtggtatcac aaggttttat gatgaggagg aaatgtatgt 11160
ggggaaccat tttctgagtg tggaagtga agaatacagag agtagctgaa tgccaacgct 11220
tctatttcag gaacatggtg agttggaggt ccagctctcg ggctcagacg ggtataggga 11280
ccaggaagtc tcacaatccg atcattctga ttttcaggg catattaggt ttggggtgca 11340
aagggaagtac ttgggactta ggcacatgag actttgtatt gaaaatcaat gattggggct 11400
ggccgtgggt ctcacgctg taatctcatc actttgggag accgaagtgg gaggatggct 11460
tgatctcaag agttggacac cagcctaggc aacatggcca gaccctctct ctacaaaaaa 11520
attaaaaatt agctggatgt ggtggtgcat gcttgtggtc tcagctatcc tggaggctga 11580
gacaggagaa tcggttgagt ctgggagttc aaggctacag ggagctgca tcacgcccgt 11640
gactccagc ctgggaaaca gagtgaact gtctcagaat ttttttaaaa aagaatcagt 11700
gatcatcca acccctgttg ctgttcaccc tgagcctgcc ttctctggct ttgttcctta 11760
gatcacatct ccatgatcca taggcctgc ccaatctgac ctcacaccgt gggaaatgcct 11820
ccagactgat ctagtatgtg tggaacagca agtgctggct ctcctccccc ttccacagct 11880
ctgggtgtgg gagggggtg tccagcctcc agcagcatgg ggaggccctt ggtcagcatc 11940

FIGURE 6H

taggtgcaa cagggcaagg gcggggctct ggagaatgaa ggctttatag ggctcctcag 12000
ggaggccccc cagccccaaa ctgcaccacc tggccgtgga caccggt 12047

FIGURE 7

cgagcgggcc ctcagcttcg gcgccagcc ccgcaaggct cccggtgacc actagagggc 60
gggaggagct cctggccagt ggtggagagt ggcaaggaag gaccctaggg ttcacggag 120
cccaggttta ctcccttaag tggaaatttc ttccccact cctccttggc tttctccaag 180
gaggggaacc aggtctctgg aaagtccggc tggggcgggg actgtgggtt caggggagaa 240
cggggtgtgg aacgggacag ggagcggta gaagggtgg gctattccgg gaagtgggtg 300
ggggagggag cccaaaacta gcacctagtc cactcattat ccagccctct tatttctcgg 360
ccgctctgct tcagtggacc cggggagggc ggggaagtgg agtgggagac ctagggtgg 420
gcttcccgac cttgctgtac aggacctga cctagctggc ttgttcccc atccccacgt 480
tagttgttgc cctgaggcta aaactagagc ccagggggcc caagttccag actgcccctc 540
ccccctccc cggagccagg gagtgggttg tgaaagggg aggccagctg gagaacaaac 600
gggtagtcag ggggttgagc gattagagcc cttgtaccct acccaggaat ggttggggag 660
gaggaggaag aggtaggagg taggggaggg ggcggggttt tgtcacctgt cacctgctcg 720
ctgtgcctag ggcgggcggg cggggagtgg ggggaccggt ataaagcgg aggcgcctgt 780
gcccgtcca cctctcaagc agccagcgcc tgcctgaatc tgttctgccc cctccccacc 840
catttcacca ccaccatg 858

FIGURE 8

aagcttccac aagtgcattt agcctctcca gtattgctga tgaatccaca gttcaggttc 60
aatggcggttc aaaacttgat caaaaatgac cagactttat attcttacac caacatctat 120
ctgattggag gaatggataa tagtcatcat gtttaaacad ctaccattcc agttaagaaa 180
atatgatagc atcttggtct tagtcttttt cttaataggg acataaagcc cacaataaaa 240
aatatgcctg aagaatggga caggcattgg gcattgtcca tgcctagtaa agtactccaa 300
gaacctatct gtataactaga tgacacaatg tcaatgtctg tgtacaactg ccaactggga 360
tgcaagacac tgcccatgcc aatcatcctg aaaagcagct ataaaaagca ggaagctact 420
ctgcaccttg tcagtgaggt ccagatacct acag 454

**This Page is Inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ BLACK BORDERS
- ☐ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES
- ☐ FADED TEXT OR DRAWING
- ☐ BLURRED OR ILLEGIBLE TEXT OR DRAWING
- ☐ SKEWED/SLANTED IMAGES
- ☐ COLOR OR BLACK AND WHITE PHOTOGRAPHS
- ☐ GRAY SCALE DOCUMENTS
- ☒ LINES OR MARKS ON ORIGINAL DOCUMENT
- ☐ REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY
- ☐ OTHER: _____

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.

THIS PAGE BLANK (USPTO)